CBR antimicrobials inhibit RNA polymerase via at least two bridge-helix cap-mediated effects on nucleotide addition

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Edited by Jeffrey W. Roberts, Cornell University, Ithaca, NY, and approved June 26, 2015 (received for review February 4, 2015)

RNA polymerase inhibitors like the CBR class that target the enzyme’s complex catalytic center are attractive leads for new antimicrobials. Catalysis by RNA polymerase involves multiple rearrangements of bridge helix, trigger loop, and active-center chains that isomerize the triphosphate of bound NTP and two Mg2+ ions from a preinsertion state to a reactive configuration. CBR inhibitors target a crevice between the N-terminal portion of the bridge helix and a surrounding cap region within which the bridge helix is thought to rearrange during the nucleotide addition cycle. We report crystal structures of CBR inhibitor/Escherichia coli RNA polymerase complexes as well as biochemical tests that establish two distinct effects of the inhibitors on the RNA polymerase catalytic site. One effect involves inhibition of trigger-loop folding via the F loop in the cap, which affects both nucleotide addition and hydrolysis of 3′-terminal dinucleotides in certain backtracked complexes. The second effect is trigger-loop independent, affects only nucleotide addition and pyrophosphorolysis, and may involve inhibition of bridge-helix movements that facilitate retractive triphosphate alignment.

CBR inhibitors | RNA polymerase | transcription inhibition | X-ray crystallography

Bacteria harbor a single RNA polymerase (RNAP) enzyme (subunit composition αββ′ω, ~400 kDa) that performs all transcription in the cell. The bacterial RNAP is a proven target for antimicrobials. The rifamycins (Rifs), which bind to and inhibit the bacterial RNAP (1–3), are potent, broad-spectrum antimicrobials and are the lynchpin of current tuberculosis therapy (4). Nevertheless, the emergence of multidrug-resistant pathogens highlights the importance of discovering novel antimicrobials (5). New RNAP inhibitors can also be used as tools to probe transcriptional mechanisms.

Artsimovitch et al. (6) described a new class of bacterial RNAP inhibitors, the CBR compounds. Single amino acid substitutions in the RNAP β and β′ subunits that conferred resistance to CBR compounds (CBR8) defined a pocket near the RNAP surface bounded by β′ residues of the F loop and the N-terminal part of the bridge helix (BH), and β residues neighboring fork-loop 2 (FL2) and the βDII loop, all RNAP structural elements shown to play important roles in the enzyme’s nucleotide addition cycle (NAC) (7–20). The genetically defined CBR site is ~31 Å from the RNAP active site Mg2+ ion and is also distinct from the binding sites of other well-characterized bacterial RNAP inhibitors (SI Appendix, Table S1). Inhibition of RNAP by the CBR compounds appears to be mechanistically distinct as well; the CBR compounds allosterically inhibit known catalytic activities of the RNAP active site preferentially at pause sites but have lesser to no effects on translocation (6, 21).

The prototype CBR compound [3-(trifluoromethyl)-N-phenylbenzamidoxime, designated CBR703] (Fig. 1 A and B), identified by screening a library of compounds for inhibition of Escherichia coli RNAP, yielded 50% inhibitory concentration (IC50) in the 10–20 μM range in in vitro assays. More potent variants, CBR9379 (IC50 of 0.3 μM) (Fig. 1C) and CBR9393 (IC50 of 2.5 μM) (Fig. 1D) were also synthesized and characterized (6). In addition to inhibiting RNAP from Gram-negative Escherichia coli, the CBR compounds inhibit the RNAP from a Gram-positive organism (Staphylococcus aureus) (22, 23). The CBR compounds do not significantly inhibit human RNAP II (IC50 > 200 μM), Thermus aquaticus, or Thermus thermophilus RNAPs. Further characterization of the CBR compound inhibition mechanism and improvement of CBR compound potency has been hampered by a lack of structural information on CBR/RNAP complexes.

In this work, we determine crystal structures that detail CBR/E. coli RNAP interactions. The structures confirm the CBR binding site defined by CBR8 mutants, provide a structural framework to investigate details of the inhibition mechanism, and set the stage for structure-based design of CBR analogs with improved properties. CBR effects on the reactions of nucleotidyl transfer and hydrolytic transcript cleavage by the RNAP catalytic site provide insights into the enzyme’s catalytic mechanism.

Results

Crystal Structures of CBR/E. coli RNAP–Holoenzyme Complexes.

To provide a structural basis for understanding CBR inhibition of RNAP, we determined crystal structures of E. coli RNAP–holoenzyme with CBR703 (3.7 Å resolution), CBR9379 (4.0 Å), and CBR9393 (3.7 Å) (Fig. 1 and SI Appendix, Fig. S1 and Table S1).

Significance

The multisubunit RNA polymerases (RNAPs) are complex molecular machines that control catalysis through concerted conformational changes of conserved structural modules surrounding the active site. Central to these modules is the bridge helix (BH). The nature of these conformational changes and their detailed roles in the different steps of the RNAP nucleotide addition cycle are central issues in understanding the structural basis of RNAP catalytic activity. We report crystal structures of Escherichia coli RNAP complexes with a class of small molecule inhibitor (CBR inhibitors) and biochemical tests that establish two distinct effects of the inhibitors on the RNAP catalytic site. These results provide insights into the enzyme’s catalytic mechanism.


The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: The crystallography, atomic coordinates, and structure factors have been deposited in the Protein Data Bank, www.pdb.org (PDB ID codes 4XSX, 4XSY, and 4XSZ).

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1502368112/-/DCSupplemental.
S2). In each case, unbiased difference Fourier maps revealed unambiguous electron density for the corresponding CBR compound consistent with the genetically defined site (Fig. 1 B–D). The electron density for CBR703 was relatively symmetric (reflecting the pseudosymmetric structure of CBR703), making determination of its orientation ambiguous at 3.7 Å resolution (Fig. 1 B). To confirm the binding orientation of CBR703, we synthesized 3-bromo-N-phenylbenzamidoxime (CBR703-Br) (Fig. 1 B). CBR703-Br was active in in vivo and in vitro assays, albeit with reduced potency (SI Appendix, Fig. S2). We collected X-ray diffraction data from CBR703-Br/E. coli RNAP–holoenzyme crystals (3.95 Å resolution) (SI Appendix, Table S2). The CBR703-Br/RNAP complex exhibited electron density for CBR703-Br matching the electron density in the CBR703/RNAP complex. In addition, an anomalous difference Fourier map revealed a single Br peak in the expected position for the Br atom of CBR703-Br in one of the two possible CBR703 orientations (Fig. 1 B). The observed binding orientation of CBR703 matches the orientation of the equivalent chemical moieties contained within CBR9379 and CBR9393 (Fig. 1). These results confirm the binding positions and orientations of each of the CBR compounds.

Conformation of CBR Compounds. Delocalized electrons over the \( \text{N} = \text{C} \rightarrow \text{N} \) bonds of CBR703 and CBR9379 yield a peptide bond-like character, with a planar configuration of the amidoxime moiety that could exist in cis or trans stereoisomers (SI Appendix, Fig. S3). CBR703 and CBR9379 bind RNAP in the cis stereoisomer (Fig. 1 B and C). This is consistent with the fixed orientation of the benzyl rings in CBR9393 (Fig. 1 D).

The amidoxime hydroxyl group could also occur in two stereoisomers, syn and anti (SI Appendix, Fig. S3). Electron density for the hydroxyl group is poorly defined, suggesting both stereoisomers may be present. There are no polar groups nearby from the RNAP that could potentially make favorable interactions with the hydroxyl group in either conformation.

CBR/RNAP Interactions. CBR703 binds in a small hydrophobic pocket in the RNAP structure surrounded by structural elements known to be critical for RNAP function (Fig. 2 A). This includes the σ70 subunit (present in the structure) and the β′-F loop (orange), the β′-BH (magenta). The atomic model for CBR703 is shown (green carbon atoms) along with the unbiased \( \text{F}_o - \text{F}_c \) map (green mesh, contoured at 3σ) and the Br anomalous Fourier difference peak (red mesh, contoured at 4.5σ). CBR9379 is a more easily synthesized, close analog of CBR9379 that was used in some experiments but not for crystal structure determination (Methods). (D, Left) Chemical structure of CBR9379 (6). (Right) Zoom-in of CBR binding pocket. The atomic model for CBR9379 is shown (green carbon atoms) along with the unbiased \( \text{F}_o - \text{F}_c \) map (green mesh, contoured at 3σ). CBR9393 is a more easily synthesized, close analog of CBR9379 that was used in some experiments but not for crystal structure determination (Methods). (D, Left) Chemical structure of CBR9393 (6). (Right) Zoom-in of CBR binding pocket. The atomic model for CBR9393 is shown (green carbon atoms) along with the unbiased \( \text{F}_o - \text{F}_c \) map (green mesh, contoured at 3σ).
elements of the β subunit that link FL2 and βDII, and the β′ subunit F loop and BH. CBR703 interacts directly with residues of the β' F loop and BH (Fig. 2A).

The CBR703-like moiety of CBR9379 binds in essentially the same manner as CBR703 and makes essentially identical interactions with the RNAP. The dichloroanilino moiety of CBR9379 fits into a narrow groove extending from the trifluoromethylphenyl ring and establishes more extensive nonpolar interactions with β residues H551, P552, and G640 and β′ residues K749 and P750. Nonpolar interactions are also established with additional residues βD444 and β′D551 (Fig. 2B).

Similarly, the CBR703-like moiety of CBR9393 makes the same set of RNAP contacts as CBR703 and the aminoethylpiperazine fits into the same narrow groove extending from the trifluoromethylbenzyl ring, establishing more extensive nonpolar interactions with β residues H551 and P552 and β′ residue P750. Additionally, βD444 makes a polar interaction with the protonated piperazyl nitrogen of CBR9393 (Fig. 2C).

Almost all of the interactions between the CBR compounds and RNAP are nonpolar in nature; only βS642 makes a polar interaction with a bridging nitrogen in each compound, and βD444 makes a polar interaction with CBR9393 (Fig. 2).

The CBR compounds interact directly with six out of the nine positions at which RNAP substitutions conferred CBRR (6) (SI Appendix, Table S3), and the effects of these six substitutions can be rationalized by examination of the structures. Substitution of βR637 with C or S (βR637C/S) or βP552L would remove nonpolar contacts with the CBR compounds. The βP552L substitution would remove polar interactions and also likely alter the conformation of the RNAP backbone near the CBR binding pocket. The βS642F substitution would remove a polar interaction and also introduce a steric clash with the CBR compounds,
whereas βS642P would remove a polar interaction and would likely alter the conformation of the RNAP backbone near the CBR binding pocket.

The βP750L and βF773V substitutions are special cases, as they make cells not just resistant to CBR compounds but dependent on their presence for growth. The βP750L substitution likely alters the conformation of the F loop. βF773 makes extensive nonpolar contacts with the CBR compounds (Fig. 2), and its substitution with the smaller V would remove many of these contacts and would alter the shape of the binding pocket. The basis for CBR703 dependence introduced by these two substitutions is difficult to understand without additional studies.

Alterations at three RNAP residues that do not make direct contact with the CBR compounds also confer CBR-resistant activity (Fig. 3B, βP560L, βE562V, and a duplication of βK639 (6)). The structures also provide insight into these findings, but the explanations are more speculative. βP560 makes direct van der Waals contact with β’F773, which in turn makes extensive nonpolar interactions with the CBR compounds (Fig. 2 and SI Appendix, Fig. S4); the βP560L substitution would introduce a steric clash with βF773, causing it to alter its conformation and thereby affecting the CBR binding pocket.

The βE562V substitution is another special case that gives rise to CBR inhibitor dependence. In the RNAP structure, βE562 is completely buried from solvent but participates in a conserved, buried network of polar interactions with βT525, βS662, and βR687 (SI Appendix, Fig. S4). The βE562V substitution would disrupt this buried network of polar interactions and would be predicted to disrupt the RNAP structure in this region. The βK639 duplication likely alters the conformation of the β loop containing G640, E641, and S642, all residues that interact with the CBR compounds.

Selectivity of CBR Compounds Toward Different RNAPs Is Explained by the CBR/RNAP Crystal Structures. The CBR compounds inhibit E. coli RNAP and also show activity against S. aureus RNAP (6, 22, 23), but they have little to no activity against T. aquaticus or T. thermophilus RNAPs. Although the BH is highly conserved among bacterial RNAPs (80% identical between E. coli and thermus RNAPs) and each of the E. coli RNAP BH residues that interact directly with the CBR compounds is identical in thermus (Fig. 3A), the F-loop sequences are more diverse (52% identical between E. coli and thermus). Indeed, swapping F-loop sequences between different bacterial RNAPs can confer distinct transcription elongation properties that correspond to the source of the F-loop sequence (8). The F-loop residue E. coli β755 forms a part of the CBR binding pocket and makes direct, nonpolar interactions with the CBR compounds (Fig. 2). The corresponding residue in both T. aquaticus and T. thermophilus RNAPs is βF1053; the Phe side chain fills the CBR binding pocket (Fig. 3B), suggesting that the CBR compounds do not inhibit thermus RNAPs because they cannot bind to the altered CBR binding pocket.

An additional structural difference between the E. coli and thermus RNAPs may have an impact on CBR binding, but the significance is less clear. As noted by Malinen et al. (21), in all of the available E. coli RNAP structures [which, to date, all come from the same crystal form (24–26)], the side chain of the BH residue β’H777 points toward the C terminus of the BH and stacks on βP750 at the tip of the F loop (Figs. 2 and 3C). In contrast, in dozens of crystal structures of thermus RNAPs in a wide variety of functional states and crystal forms, the side chain of the corresponding residue, β’H1075, points up toward the N terminus of the BH, although it still stacks on F-loop βP1048 (the two F loops are in nearly identical conformations) (Fig. 3C). The latter conformer of β’H1075 in the thermus RNAPs creates a steric clash with the CBR compounds (Fig. 3C). The different
conformers of this BH. His residue observed in \textit{E. coli} and \textit{thermus} RNAP structures raises two distinct possibilities: (i) The side chain alternates between upward- and downward-facing rotamers depending on the functional state of the RNAP, or (ii) the two conformers represent a persistent structural difference between \textit{E. coli} and \textit{thermus} RNAPs. The first hypothesis is difficult to conclude, as many different structures of \textit{thermus} RNAPs in different conformations and functional states do not reveal a different conformer of this residue. The second hypothesis could also help explain why the CBR compounds are inactive against \textit{thermus} RNAPs, but this conclusion would also be tenuous, as \textit{E. coli} RNAP structures are currently only available from one crystal form.

The CBR compounds also show no activity against eukaryotic RNAP II (6). The RNAP II F loop includes a 20-residue insert compared with bacterial RNAPs (Fig. 3A) that covers and seals off the putative CBR binding pocket (Fig. 3D), explaining the lack of CBR compound activity against eukaryotic RNAP II.

**CBR Inhibition of RNAP Nucleotide Addition Is Partially Independent of the Trigger Loop.** The RNAP NAC is governed by alternate opening and closure of the active site by a mobile structural element of the \( \beta' \) subunit called the trigger loop (TL). Translocation of the elongation complex (EC) along the DNA template, as well as entry and binding of the NTP substrate in the active site, is facilitated by an open active site with an unfolded TL (27). TL contacts with the correct NTP substrate stabilize the folded TL, which closes the active site. TL contacts to NTP and RNAP then participate in an active-site rearrangement that shifts the NTP triphosphate into reactive alignment and stimulates catalysis by \( \sim 10^4 \) (28–30).

The tip of the folded TL is accommodated in a pocket surrounded by conserved structural elements of the RNAP, including the F loop that immediately precedes the BH in the \( \beta' \) sequence. Alterations to the F loop have profound effects on the RNAP NAC, likely through effects on TL folding (8–10).

The CBR compounds make direct interactions with residues of the F loop (\( \beta'K749, \beta'P750, \beta'D751 \), and \( \beta'I755 \)) (Fig. 2), so we hypothesized that the binding of the CBR compounds may influence TL folding. To test this idea, we monitored TL folding by the formation of a disulfide bond in a Cys-pair reporter engineered to report the folded TL conformation (\( \beta'937–736 \) cross-link) (31). Addition of CBR9379 significantly decreased the formation of the 937–736 cross-link in a previously used model EC, with an apparent \( K_i \) of \( \sim 5 \) \( \mu \)M (Fig. 4A).

Function of CBR9379 concentration in the (\( \beta'937–736 \))-RNAP. The \textit{Inset} shows the nonreducing SDS/PAGE gel that detects a decrease in formation of the cross-linked \( \beta' \) as the CBR9379 concentration was increased from 0 to 40 \( \mu \)M. (B) CBR9379 inhibits transcript elongation by the WT- and \( \Delta TL \)-RNAP. (Top) The EC scaffold used in the elongation assay. The 5'-(\( ^{32} \)P)-labeled RNA (C17, shown in red capital letters) can be extended 9 nt (to A-26, lowercase, underlined text) upon addition of GTP and ATP. (Middle) Diagram illustrating the experimental protocol. (Bottom) Plot showing the effect of CBR9379 on the elongation profiles of WT- and \( \Delta TL \)-RNAP. With or without 50 \( \mu \)M CBR9379, elongation of C17 was single-exponential for the WT-RNAP (on average, \( \sim 0.05 \) s\(^{-1} \) and \( \sim 0.009 \) s\(^{-1} \), respectively) but was complex for WT-RNAP (on average, \( \sim 0.05 \) s\(^{-1} \) and \( \sim 0.009 \) s\(^{-1} \), respectively). (C) Effect of CBR9379 titration on the elongation profiles of WT- and \( \Delta TL \)-RNAPs. The EC scaffold used in the elongation assay is the same as in Fig. 4B. (Top) Diagram illustrating the experimental protocol. (Bottom) Plot showing fraction EC (\( >RNA17 \)) extended versus CBR9379 concentration. Note that some residual activity remains even at the highest CBR9379 concentrations for the \( \Delta TL \)-RNAP.

\begin{figure}[h]
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\caption{CBR9379 inhibits TL folding in an EC but still inhibits \( \Delta TL \)-RNAP. (A) CBR9379 inhibits formation of the folded TL in an EC. (Top) Diagram illustrating the location of cysteine substitutions at position 937 and 736 on the \( \beta' \) subunit that cross-link in the folded TL conformation (\( \beta'937–736 \)) and the model EC scaffold used in prior studies (31). (Middle) The experimental protocol. (Bottom) Plot showing the disulfide cross-linking profile as a function of CBR9379 concentration in the (\( \beta'937–736 \))-RNAP. The \textit{Inset} shows the nonreducing SDS/PAGE gel that detects a decrease in formation of the cross-linked \( \beta' \) as the CBR9379 concentration was increased from 0 to 40 \( \mu \)M. (B) CBR9379 inhibits transcript elongation by the WT- and \( \Delta TL \)-RNAP. (Top) The EC scaffold used in the elongation assay. The 5'-(\( ^{32} \)P)-labeled RNA (C17, shown in red capital letters) can be extended 9 nt (to A-26, lowercase, underlined text) upon addition of GTP and ATP. (Middle) Diagram illustrating the experimental protocol. (Bottom) Plot showing the effect of CBR9379 on the elongation profiles of WT- and \( \Delta TL \)-RNAP. With or without 50 \( \mu \)M CBR9379, elongation of C17 was single-exponential for the WT-RNAP (on average, \( \sim 0.05 \) s\(^{-1} \) and \( \sim 0.009 \) s\(^{-1} \), respectively) but was complex for WT-RNAP (on average, \( \sim 0.05 \) s\(^{-1} \) and \( \sim 0.009 \) s\(^{-1} \), respectively). (C) Effect of CBR9379 titration on the elongation profiles of WT- and \( \Delta TL \)-RNAPs. The EC scaffold used in the elongation assay is the same as in Fig. 4B. (Top) Diagram illustrating the experimental protocol. (Bottom) Plot showing fraction EC (\( >RNA17 \)) extended versus CBR9379 concentration. Note that some residual activity remains even at the highest CBR9379 concentrations for the \( \Delta TL \)-RNAP.
\end{figure}
that if the inhibition of TL folding by the CBR compounds was the sole mechanism for the inhibition of RNAP, then the CBR compounds would not inhibit ΔTL-RNAP. However, the TL is not required for all transcription inhibition by CBR9379 in an EC that enables measurement of nucleotide addition rates at saturating NTP (Fig. 4B and SI Appendix, Fig. S5A): of ~120-fold CBR inhibition in WT-RNAP, ~fivefold remained in ΔTL-RNAP. Moreover, the concentration dependence of CBR9379-mediated inhibition of WT and ΔTL-RNAPs was similar (Fig. 4C and SI Appendix, Fig. S5B). We conclude that although the CBR compounds interfere with TL folding, which is obligatory for efficient catalysis of phosphodiester bond formation, the inhibition of TL folding is not the sole mechanism through which the CBR compounds inhibit the RNAP NAC. The CBR compounds also either inhibit a step distinct from TL action that becomes rate-limiting in ΔTL-RNAP or, more likely, inhibit an active-site rearrangement linked to the BH that acts in concert with the TL to aid catalysis (Discussion).

**CBR Inhibition of RNAP Intrinsic Transcript Cleavage Is TL-Dependent.**

In addition to phosphoryl transfer activity required for transcription, RNAP possesses hydrolytic activity against nascent RNAs aided by proton transfer to a TL His in some cases and by Gre cleavage factors in other cases (33, 34). CBR703 was shown previously to inhibit TL-independent, Gre-factor–dependent transcript cleavage, presumably by interfering with Gre-factor binding, but the TL-dependent, factor-independent intrinsic cleavage reaction was not investigated (6).

To ask if CBR compounds affect the intrinsic hydrolytic activity of RNAP, we tested CBR9379 inhibition of intrinsic cleavage on scaffolds that force 1-nt backtrack states with a noncomplementary 3′ nt. Because intrinsic cleavage is known to be sensitive to the nucleotide at the RNA 3′ end (35), we used two different backtracked scaffolds, a 1-nt A backtrack and a 1-nt U backtrack (Fig. 5 and SI Appendix, Fig. S6). Intrinsic cleavage of the 1-nt A backtrack was particularly effective (50% cleavage after ~5 min, compared with ~22 min for the 1-nt U backtrack). Uniquely efficient cleavage of 1-nt A-backtracked transcripts has been noted previously (35) and is thought to be accelerated by the TL His residue (36). In the presence of a saturating concentration of CBR9379, cleavage of the 1-nt A-backtracked complex was inhibited much more significantly than the 1-nt U-backtracked complex, so that the cleavage rates for both complexes became nearly equal (50% cleavage after ~50 min) (Fig. 5).

Deletion of the TL had little effect on cleavage of the 1-nt U backtrack but slowed cleavage of the 1-nt A backtrack to a rate very similar to cleavage of the 1-nt U backtrack (Fig. 5). This result suggests that the TL does not play a role in intrinsic cleavage when the backtracked nt is a U, unlike its important role in the hyperefficient intrinsic cleavage when the backtracked nt is an A. Structural studies suggest that a backtracked U often resides in the E site (as observed in mismatched RNAPII complexes (37), whereas a backtracked 3′ purine resides in the recently defined P site (38, 39). Occupancy of either the E or P site appears to be incompatible with TL folding, but we note the following: (i) the backtracked states seen in structural studies may not correspond to the configuration in which cleavage occurs, and (ii) the structure of the TL required for TL His participation in backtracked A cleavage (33) may not correspond to the structure of the folded TL needed for stimulation of the NAC. Our results are consistent with the suggestion that the 3′ U in the E site could interfere with the TL conformation required for TL His participation in the intrinsic cleavage reaction. Consistent with this view, the intrinsic cleavage activity of WT-RNAP was sensitive to CBR9379 but that of the ΔTL-RNAP was essentially insensitive (Fig. 5 and SI Appendix, Fig. S6). Thus, inhibition of intrinsic cleavage by CBR9379 appears to be nearly completely mediated through the effect of CBR9379 on blocking TL function (Fig. 4A).

**The CBR Compounds Inhibit Pyrophosphorolysis in a TL-Independent Manner.**

To probe the idea that CBR compounds affect RNAP phosphoryl transfer but not hydrolytic reactions via a TL-independent path, we examined pyrophosphorolysis. Pyrophosphate drives the RNAP NAC in reverse, yielding NTPs during processive shortening of the RNA transcript (40). We investigated the effect of CBR9379 on pyrophosphorolysis of pretranslocated ECs (C10) formed on a previously characterized scaffold (41) that yields product CTP (Fig. 6A and SI Appendix, Fig. S7). CBR9379 inhibited pyrophosphorolysis (Fig. 6 and SI Appendix, Fig. S7).
As expected for the reverse reaction of the NAC, deletion of the TL dramatically slowed pyrophosphorolysis (a higher concentration of pyrophosphate and longer incubation time was required for a similar level of pyrophosphorolysis) (SI Appendix, Fig. S7). However, like nucleotide addition, normalized inhibition curves revealed similar inhibition of pyrophosphorolysis by CBR9379 in WT- and ΔTL-RNAPs (SI Appendix, Fig. S6B). Thus, the TL-independent component of CBR inhibition observed for nucleotide addition, whether affecting the same or a different NAC step, as the TL also is evident in the reverse phosphoryl transfer reaction.

Addition of pyrophosphate also stimulates intrinsic exonucleolytic cleavage in TEC10C (42), a reaction yielding pC (SI Appendix, Fig. S7). Thus, we could monitor CBR9379 inhibition of exonucleolytic cleavage in the same experiment used to monitor pyrophosphorolysis. Intrinsic exonucleolytic cleavage was inhibited by CBR9379 (Fig. 6C). Strikingly, deletion of the TL led to a complete loss of sensitivity of the exonucleolytic reaction to CBR9379 (Fig. 6C).

Together, these results support the view that CBR affects phosphoryl transfer through both TL-dependent and TL-independent routes but affects hydrolysis only through a TL-dependent path.

Discussion

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Discussion

The multisubunit cellular RNAPs are complex molecular machines that control catalysis through concerted conformational changes of structural modules surrounding the active site. These structural modules include the F loop, the TL, and the BH. The nature of these conformational changes, their coordination, and their detailed roles in the different steps of the reactions of RNA synthesis and degradation catalyzed by the RNAP active site (42) remain central issues in understanding the structural basis of RNAP catalytic activity.

The BH, a long α-helix of the β′ subunit that traverses across the RNAP active site, separating the RNAP nucleic acid binding cleft from the secondary channel, plays a central role by butressing the F loop and TL. The BH is one of the most highly conserved elements of the RNAP (Fig. 3A), highlighting its key role in RNAP catalysis. Because the BH has been observed in either straight or kinked conformations in different RNAP crystal structures, it has been proposed that cycling of the BH between these two conformations is an integral part of the RNAP NAC (7, 15, 17, 43, 44).

Transcription inhibitors can be powerful tools to study RNAP conformational dynamics. Some RNAP inhibitors, such as streptolydigin (Stl), are thought to function by interfering with TL and/or BH conformational changes (30, 45). Other inhibitors have been proposed to function by interfering with conformational changes of the RNAP clamp domain (46, 47).

Our structural results establish that the CBR compounds bind a small, preformed hydrophobic pocket in the RNAP structure surrounded by structural elements known to be critical for RNAP function (Fig. 2), including elements of the β subunit that link FL2 and βDII, and the β′ subunit F loop and BH. The mobile RNAP active-center structural modules are either contacted...
directly by the bound CBR compounds (F loop and BH), or CBR binding affects their dynamics (TL). The CBR binding pocket is far from the RNAP active site (31 Å), but CBR compound binding effectively inhibits all known catalytic activities of the RNAP active site (6), including intrinsic transcript cleavage (Fig. 5). The CBR compounds do not compete with substrate binding and also do not principally affect translocation (6, 21), reflecting an allosteric inhibitory mechanism.

The results of the present study provide insights into the catalytic mechanism of RNAP. By virtue of specific contacts with the F loop, the CBR compounds inhibit TL folding (Fig. 4A). However, the TL is not required for CBR-mediated suppression of nucleotide addition (Fig. 4 B and C), pointing to an additional mechanism for inhibition. Similarly, CBR compounds inhibited pyrophosphorylation even when the TL was deleted (Fig. 6C). Thus, although interference with TL folding may contribute to CBR-mediated inhibition, a key basis for CBR inhibition of nucleotide addition involves an aspect of RNAP catalysis that is TL-independent. This feature of RNAP catalysis likely involves a conformational rearrangement that is blocked by CBR compound binding and therefore would likely involve rearrangements of RNAP elements directly contacted by the CBR compounds (the N-terminal segment of the BH, the F loop, and nearby elements of the β subunit) (Fig. 2).

Intrinsic cleavage is promoted by the TL, at least for the 1-nt A-backtracked EC (Fig. 5A), but intrinsic cleavage by the ΔTL-RNAP is not further inhibited by the presence of CBR9379 (Figs. 5 and 6), indicating that the putative conformational rearrangement inhibited by the CBR compounds, while being important for nucleotide addition, is not important for intrinsic cleavage.

Essential conformational changes around the N-terminal part of the BH (where CBRs bind) (Fig. 2) have previously been proposed (48). The most compelling evidence for conformational changes in the N-terminal region of the BH comes from Prosancing mutagenesis of the entire BH sequence of Methanothermobacter jannaschii RNAP; Pro substitutions at every position of the BH drastically lower transcription activity, except at two positions, corresponding to E. coli Φ1777P (defining the BH N-terminal hinge; BH-Hi) and Φ793P (defining BH-II) (Fig. 3A), where Pro substitutions strikingly increase activity (48).

Therefore, if substitution at two specific positions increase transcription activity, it implies that the helical structure of the BH may normally (in WT-RNAP) be transiently disrupted (possibly kinked) at these sites during the NAC and that the kinked conformations aid one or more steps of the NAC. Kinking has been directly observed for the BH-H2C: Crystal structures of bacterial RNAP generally show two distinct conformations of the BH, either straight or kinked. In structures with a kinked BH, the kink occurs exactly at the position defined as the BH-H2C. No such BH conformational changes have been observed at the BH-H2C. However, crystal structures trap static, thermodynamically stable structures. It is possible the putative alternative conformation of the BH kinked at BH-H2C is unstable and occurs only transiently during the NAC.

Our results support the hypothesis that the CBRs inhibit phosphofuryl transfer reactions catalyzed by RNAP in part by preventing the kinked BH conformation at the BH-H2C. Another recently described inhibitor, salinamide, also binds near BH-H2C, although to a distinct site (49). It seems likely that salinamide acts by a mechanism similar to CBR inhibitors by inhibiting TL-folding and also by affecting BH-H2C kinking.

Given that the TL-independent inhibition of catalysis by CBR compounds is observed only for reactions involving triphosphate (nucleotide addition and pyrophosphorylation) and the observation that NTPs bind in the RNAP active site in both active (insertion) and inactive (preinsertion) configurations of the triphosphate (28), it is tempting to speculate that the triphosphate rearrangement may be facilitated by linked conformational changes in the active site and BH-H2C. If such motions are required to move the triphosphate into reactive position relative to the catalytic Mg2+ ions (or to move pyrophosphate in the case of pyrophosphorylase), then the TL-independent effect of CBR compounds could be accounted for by inhibition of the BH movements or movements of surrounding parts of RNAP coupled to the BH movements. Such an effect of the BH could stabilize the transition state additively with nucleotide positioning by TL folding and thus could contribute to catalysis in WT-RNAP, although we cannot currently rule out the possibility that a slow BH-dependent effect on catalysis is unmasked in ΔTL-RNAP. It also is possible that a TL- or BH-dependent effect contributes on the relevant time scale at some template positions more than others, which could explain why CBR compounds affect WT-RNAP at some template positions like pause sites more than others (6, 21). Indeed, we observed more uniform effects of CBR compounds in ΔTL-RNAP than WT-RNAP (e.g., SI Appendix, Fig. 5B). Further experimentation will be necessary to test these ideas.

Materials and Methods

CBR Compounds. CBR703 (Fig. 1A), CBR9379 (Fig. 1C), and CBR9393 (Fig. 1D) were synthesized as described previously (6, 22, 23). CBR703-Br was synthesized described forms 4-bromo-N-phenylbenzamide (30) substituting 3-bromo-N-phenylbenzamide for 4-bromo-N-phenylbenzamide. CBR93795 was synthesized as described for CBR9379 but substituting thiophosphogene for phosgene.

Crystallization of CBR/E. coli RNAP-Holoenzyme Complexes. Components of E. coli RNAP-holoenzyme (E. coli ΔCTD-RNAP core, Δ1.1ε20) were prepared as previously described (26). Before crystallization, aliquots of purified components were thawed on ice and buffer-exchanged to crystallization buffer (20 mM Tris-HCl, pH 8.0, 0.2 M NaCl, 5 mM DTT) to remove residual glycerol from storage buffer. E. coli RNAP-holoenzyme was formed by adding a 1.2-fold molar excess of Δ1.1ε20 to the ΔCTD-core RNAP and incubated it at room temperature (RT) for 15 min. CBR/RNAP-holoenzyme complexes were formed by adding a 20-fold molar excess of the CBR compound (from an 80 mM stock solution in ethanol) to the holoenzyme and incubated it at RT for 15 min. The final concentration of holoenzyme was adjusted to 40–60 μM. The CBR/RNAP-holoenzyme complex crystals were grown and cryo-protected as described for crystals of RNAP-holoenzyme (26).

Data Collection, Refinement, and Model Building. X-ray diffraction data were collected at Brookhaven National Laboratory National Synchrotron Light Source (NSLS) Beamline X29. Data were integrated and scaled with the HKL-2000 package (51). The molecular replacement was performed using the holoenzyme structure (SI Appendix, Table S2). The initial difference maps calculated after rigid body refinement (52) of the holoenzyme showed clear difference density corresponding to each CBR compound (Fig. 2 B–D). The models were improved using rigid body refinement of 20 individual domains using PHENIX (52), followed by iterative cycles of manual building with COOT (53). The atomic coordinates and restraints for each CBR compound were generated using electronic ligand builder and optimization workbench (eLBOW) tools in PHENIX (54). Each CBR compound was modeled to fit the strong positive (Fo – Fo) Fourier difference density, and the resulting CBR/RNAP-holoenzyme models were further refined using defomable elastic network refinement with noncrystallographic symmetry restraints using CNS 1.3 performed on the SBGrid cluster (55–57). The model was manually inspected and corrected based on Ramachandran restraints, followed by final positional and B-factor refinement in PHENIX (52).

Disulfide Cross-Linking Assay. ECs were formed by incubating 1 μM E. coli core RNAP, bearing the β397C–736C substitutions (31), and 2 μM EC scaffold (Fig. 4A) in transcription buffer (TB) (50 mM TrisCl, pH 8.0, 20 mM NaCl, 10 mM MgCl2, 0.1 mM EDTA, 2.5 μg acetylated BSA/mL) for 15 min at RT. CBR9379 was titrated into the ECs (0–40 μM) and incubated for 5 min at RT. Disulfide cross-linking was initiated by incubating the ECs with 2.5 mM cystamine and 2 mM DTT for 1 h at RT. Cross-linking was quenched by addition of 20 mM iodoacetamide for 5 min at RT. Formation of the disulfide bond was evaluated by nonreducing SDS/PAGE on a 4–15% precast Phastgel (GE Pharmacia) followed by coomassie staining and densitometry using ImageJ.
In Vitro Transcription Elongation Assay. Scaffold was prepared by mixing 500 nM 5'-32P-labeled RNA, 1000 nM template DNA, and 2000 nM nontemplate DNA (Fig. 4B) in reconstitution buffer (20 mM Tris-Cl, pH 8.0, 20 mM NaCl, 0.1 mM EDTA), heated to 95°C for 5 min, followed by rapid cooling to 4°C, and then slow cooling to RT in 2 °C2 min steps (31). ECs were reconstituted by incubating 50 nM scaffold either with 150 nM WT-RNAP or ATL-RNAP in TB for 15 min at 37°C. Following reconstitution, ECs were incubated without or with 50 μM CBR9379 at 37°C for 5 min before extending by 9 nt by adjusting to 1 mM each GTP and ATP. Samples were taken for times of 5 s or less using a Kintek quench-flow apparatus, quenched with 2 M HCl (40 μL), and immediately neutralized to pH 8.0 by addition of 3 M Tris base (40 μL). The remaining RNA-17 fraction in each lane was calculated and plotted versus time to generate plots for WT- and ATL-RNAPs (Fig. 4A and B).

Intrinsic Transcription Cleavage. Scaffolds for intrinsic transcription cleavage were prepared by assembling 5'-32P-labeled RNA that has either a t-1 t-A or t-1 U mismatch at the 3' end, with template DNA and nontemplate DNA (Fig. 5A and B) in reconstitution buffer following the protocol described above. ECs were prepared by incubating 50 nM scaffold either with 150 nM WT-RNAP or ATL-RNAP in cleavage buffer (20 mM Tris-Cl, pH 8.0, 20 mM NaCl, 0.1 mM EDTA) for 25 min at 37°C. Reactions were then treated without or with 100 μM CBR9379 for 5 min at 37°C. Intrinsic cleavage reactions were initiated by adding 20 mM MgCl2 to the ECs, and samples were removed at the indicated times (Fig. 5) and quenched by addition of an equal volume of 2x stop buffer. Transcription products were resolved by denaturing 20% polyacrylamide gel electrophoresis. Gels were quanitied as described above, and fraction remaining RNA18 or RNA18 in each lane was calculated and plotted versus time to generate plots for WT- and ATL-RNAPs (Fig. 5A and B).

Pyrophosphorylation and Exonucleolytic Transcript Cleavage. RNA and template-stand DNA oligonucleotides (1 μM each) (Fig. 6A) in 10 μL of TB (20 mM Tris-HCl, 10 mM MgCl2, 100 mM NaCl) were placed in warm water and allowed to cool down from 40°C to RT in about 30 min followed by addition of the mixture to the equivalent amount of RNAP immobilized to NTA agarose through a His65-tag. After 20 min of agitation at RT, an equivalent amount of RA nontemplate strand was added and agitation continued for another 20 min. After extensive washing with TB, the beads were suspended in 10 μL of TB, supplemented with 1 μL of (32P)CTP (3,000 Ci/mmol, 1 mCi/L), and left at RT. Incubation time was 5 min for WT- and 4 h for ATL-RNAP, respectively. The excess of nonincorporated radioactive material was removed by washing with TB mixing MgCl2. The labeled TECs thus obtained were incubated at various conditions (10 μL reaction volumes) specified in Fig. 6B. Reactions were stopped by addition of an equal volume of the mixture containing 12 M urea and 50 mM EDTA. The samples (1 μL) were applied on a polyethylenimine cellulose plate (Sigma-Aldrich), dried, washed with water, dried again, and subjected to TLC using 0.5 M monobasic potassium phosphate as the developing solvent. After a ~10-cm run, the plates were dried and the products of interest quantified using a Phosphorimager.

ACKNOWLEDGMENTS. We thank Wuxian Shi at National Synchrotron Light Source (NSLS) beamline X29 for support with synchrotron data collection. This work was based, in part, on research conducted at the NSLS, supported by the US Department of Energy, Office of Basic Energy Sciences. The work at NSLS X29 was made possible by Center for Synchrotron Bionanosciences Grant P30-EB-009998, from the National Institute of Biomedical Imaging and Bioengineering. B.B. was supported by a Merck Postdoctoral Fellowship (The Rockefeller University) and National Research Service Award NHI F32 GM071354-01A. This work was supported, in part, by NIH Grant GM38660 (to R.L.) and Grants GM53759 and GM97458 (to S.A.D.).


**Table S1.** CBR703 interacts with RNAP in a site distinct from the RNAP active-site Mg$^{2+}$-ion and from known antibiotics

<table>
<thead>
<tr>
<th>CBR703 to:</th>
<th>Distance (Å)$^1$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg$^{2+}$</td>
<td>31</td>
<td>This work</td>
</tr>
<tr>
<td>Lipiarmycin</td>
<td>56</td>
<td>(1-5)</td>
</tr>
<tr>
<td>Microcin J25</td>
<td>21</td>
<td>(6, 7)</td>
</tr>
<tr>
<td>Myxopyronin</td>
<td>49</td>
<td>3DXJ, 3EQL</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>30</td>
<td>4KMU</td>
</tr>
<tr>
<td>Streptolydigin</td>
<td>23</td>
<td>1ZYR, 2A6H</td>
</tr>
</tbody>
</table>

$^1$Cα coordinates of holoenzyme/antibiotic complexes were superimposed using Pymol. The distances between the CBR703 center of mass and that of the alternative antibiotic (or residues conferring resistance, where a structure of the RNAP complex is not available) were measured.
<table>
<thead>
<tr>
<th></th>
<th>CBR703</th>
<th>CBR9379</th>
<th>CBR9393</th>
<th>CBR703Br</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Data collection</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Space group</td>
<td>P2 2 2</td>
<td>P2 2 2</td>
<td>P2 2 2</td>
<td>P2 2 2</td>
</tr>
<tr>
<td>Combined datasets</td>
<td>4</td>
<td>5</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td><strong>Cell dimensions</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a (\text{Å}))</td>
<td>187.03</td>
<td>185.39</td>
<td>184.44</td>
<td>187.56</td>
</tr>
<tr>
<td>(b (\text{Å}))</td>
<td>206.91</td>
<td>206.77</td>
<td>206.33</td>
<td>207.09</td>
</tr>
<tr>
<td>(c (\text{Å}))</td>
<td>310.04</td>
<td>310.27</td>
<td>309.61</td>
<td>308.82</td>
</tr>
<tr>
<td><strong>Wavelength (Å)</strong></td>
<td>40.07 - 3.71</td>
<td>40.36 - 4.01</td>
<td>39.93 - 3.68</td>
<td>44.7 - 3.97</td>
</tr>
<tr>
<td><strong>Resolution (Å)</strong></td>
<td>(3.84 - 3.71)(^a)</td>
<td>(4.15 - 4.01)(^a)</td>
<td>(3.81 - 3.68)(^a)</td>
<td>(4.11 - 3.97)(^a)</td>
</tr>
<tr>
<td><strong>Total reflections</strong></td>
<td>1,671,459 (134,709)</td>
<td>1,579,819 (108,343)</td>
<td>2,086,777 (168,875)</td>
<td>974,519 (76,360)</td>
</tr>
<tr>
<td><strong>Unique reflections</strong></td>
<td>126,238 (11,386)</td>
<td>96,477 (7,933)</td>
<td>113,219 (10,195)</td>
<td>101,829 (9,373)</td>
</tr>
<tr>
<td><strong>Completeness (%)</strong></td>
<td>98.9 (89.4)</td>
<td>96.0 (72.3)</td>
<td>88.5 (79.0)</td>
<td>97.6 (88.0)</td>
</tr>
<tr>
<td>(&lt;I&gt;/\sigma(I))</td>
<td>16.4 (1.3)</td>
<td>15.5 (0.84)</td>
<td>16.2 (1.08)</td>
<td>14.0 (1.84)</td>
</tr>
<tr>
<td><strong>Wilson B-factor</strong></td>
<td>0.174 (2.106)</td>
<td>0.170 (4.313)</td>
<td>0.209 (2.807)</td>
<td>0.166 (1.262)</td>
</tr>
<tr>
<td><strong>(R_{merge})</strong></td>
<td>0.1808</td>
<td>0.1751</td>
<td>0.2146</td>
<td>0.1755</td>
</tr>
<tr>
<td><strong>(R_{sy}^{b})</strong></td>
<td>0.049 (0.607)</td>
<td>0.041 (0.942)</td>
<td>0.047 (0.636)</td>
<td>0.077 (0.618)</td>
</tr>
<tr>
<td><strong>CC1/2</strong> (^c)</td>
<td>0.999 (0.573)</td>
<td>0.999 (0.59)</td>
<td>0.999 (0.453)</td>
<td>0.998 (0.735)</td>
</tr>
<tr>
<td><strong>CC</strong> (^c) (^b)</td>
<td>1.000 (0.853)</td>
<td>1.000 (0.861)</td>
<td>1.000 (0.790)</td>
<td>1.000 (0.921)</td>
</tr>
</tbody>
</table>

**Refinement**

\(R_{work} / R_{free}\) | 0.230/0.273 | 0.235/0.270 | 0.240/0.286 | 0.235/0.275 |
| \((0.336/0.397)\) | \((0.355/0.398)\) | \((0.340/0.408)\) | \((0.335/0.389)\) |
| **CC_{work}/CC_{free}\(^c\)** | 0.921/0.916 | 0.895/0.911 | 0.915/0.920 | 0.907/0.906 |
| \((0.658/0.488)\) | \((0.585/0.495)\) | \((0.546/0.413)\) | \((0.760/0.673)\) |
| **No. atoms** | 55,722 | 55,746 | 55,544 | 59,207 |
| **Protein** | 55,676 | 55,676 | 55,676 | 55,676 |
| **Ligand/ion** | 46 | 70 | 68 | 40 |
| **Water** | 0 | 0 | 0 | 0 |
| **Protein residues** | 7,084 | 7,084 | 7,084 | 7,084 |
| **B-factors** |                 |                 |                 |                 |
| **Protein** | 102.00 | 97.60 | 97.90 | 102.70 |
| **Ligand/ion** | 109.10 | 111.70 | 83.80 | 114.20 |
| **R.m.s deviations** |                 |                 |                 |                 |
| **Bond lengths (Å)** | 0.005 | 0.005 | 0.005 | 0.005 |
| **Bond angles (°)** | 1.03 | 0.97 | 1.03 | 1.03 |
| **Clashscore** | 11.30 | 11.87 | 11.67 | 14.95 |
| **Ramachandran favored (%)** | 92 | 91 | 92 | 92 |
| **Ramachandran outliers (%)** | 0.38 | 0.31 | 0.31 | 0.38 |

\(^a\)Values in parentheses are for highest-resolution shell.
\(^b\)\((8)\).
\(^c\)\((9)\).
**Table S3.** Identity and properties of *Eco tolC* mutants with altered sensitivity to CBR703 {Artsimovitch:2003bp}).

<table>
<thead>
<tr>
<th>RNAP subunit</th>
<th>Substitution</th>
<th>Phenotype&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Direct interaction with CBR703? (Y/N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β</td>
<td>P552L</td>
<td>R</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>P560L</td>
<td>R</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>E562V</td>
<td>D</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>K639 duplication</td>
<td>R</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>R637C</td>
<td>R</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>R637S</td>
<td>R</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>S642F</td>
<td>R</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>S642P</td>
<td>R</td>
<td>Y</td>
</tr>
<tr>
<td>β'</td>
<td>P750L</td>
<td>D</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>P750L/D727Y</td>
<td>S</td>
<td>Y/N</td>
</tr>
<tr>
<td></td>
<td>F773V</td>
<td>D</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>F773V/R744C</td>
<td>S</td>
<td>Y/N</td>
</tr>
<tr>
<td></td>
<td>I774S</td>
<td>R</td>
<td>Y</td>
</tr>
</tbody>
</table>

<sup>a</sup> D, dependent; R, resistant; S, sensitive
Supplementary Figure Legends

Fig. S1. Data and model quality

Plots relating data quality with model quality using the Pearson correlation coefficient (CC) analysis described by Karplus & Diederichs (9). CC1/2 (red squares) was determined from the unmerged diffraction data randomly divided in half. Since CC1/2 underestimates the information content of the data (since it's calculated by dividing the dataset in half), CC* was calculated from an analytical relation (9) to estimate the information content of the full data. CC* provides a statistic that assesses data quality as well and also allows direct comparison of crystallographic model quality and data quality on the same scale through CC_work and CC_free, the standard and cross-validated correlations of the experimental intensities with the intensities calculated from the refined model. A CC_work/CC_free smaller than CC* indicates that signal is present in the data, and that the model does not account for all of the signal (meaning it is not overfit). Plotted also are the standard <\sigma>/\sigma for the diffraction data, as well as the R_work/R_free for the refined models.

A. Data for CBR703/Eco RNAP-Δ1.1σ^70 (3.7 Å-resolution).

B. Data for CBR79379/Eco RNAP-Δ1.1σ^70 (4.0 Å-resolution).

C. Data for CBR79393/Eco RNAP-Δ1.1σ^70 (3.7 Å-resolution).
Fig. S2. Br-CBR703 inhibits RNAP.

(A) The EC scaffold 5 used for the assay of Br-CBR703 and the experimental protocol. Extension with NTPs generates a run-off transcript, whereas hydrolytic cleavage or pyrophosphorolysis generate an ApC or pC (CMP), respectively.

(B) Gel showing the effect of increasing concentrations of Br-CBR703 on the reaction products.

(C) Plots of the relative amounts of each reaction product relative to 100% in the absence of inhibitor.

Fig. S3. Stereoisomers of CBR703

Four stereoisomers of CBR703.

Fig. S4. Structural diagram to explain effects of CBR^R substitutions

The RNAP is shown as an α-carbon backbone worm (β subunit, light cyan; β'F-loop, orange; β'BH, magenta). Selected side chains are shown in stick representation with transparent CPK spheres. CBR703 is shown with green carbon atoms. A network of buried polar interactions involving βE562 are shown as dashed red lines.
Fig. S5. CBR9379 inhibits WT- and ΔTL-RNAPs.

(A) (top) The EC scaffold used in the elongation assay. The 5'-'[^32P] RNA (C17, shown in red capital letters) can be extended 9 nt (to A26; lower case, underlined text) upon addition of GTP and ATP. (middle) Diagram illustrating the experimental protocol. (bottom) Denaturing PAGE gels showing extension of the 5'-[^32P] labeled RNA17 (C17) by 9 nt as a function of time (0-32 s or 0-1024 s for WT-RNAP +/- CBR9379, 0-3840 s for ΔTL-RNAP).

(B) (top) The EC scaffold used in the elongation assay. The 5'-'[^32P] RNA (C-17, shown in red capital letters) can be extended 9 nt (to A26; lower case, underlined text) upon addition of GTP and ATP. (middle) Diagram illustrating the experimental protocol. (bottom) Denaturing PAGE showing extension of the 5'-'[^32P] labeled RNA17 (C17) by 9 nt as a function of increasing concentration of CBR 9379 (0-80 uM). NTP concentration and reaction time were optimized both for WT- and ΔTL-RNAP, so that it will span the spectrum of no inhibition to maximal CBR inhibition. For WT, reactions were quenched 10 sec after addition of 10 uM GTP and ATP and for ΔTL RNAP, 3 min after addition of 1 mM each of GTP and ATP (see reaction schemes above the gel).
Fig. S6. Effect of CBR9379 on intrinsic transcript cleavage profiles of wt- and ΔTL-RNAPs on backtracked ECs

(A) (top) Backtracked EC scaffold (1 nt A mismatch at the 3’-end to yield a 1 nt A backtracked EC) used in the intrinsic cleavage experiments. The RNA cleavage position is shown by an upward arrow below the scaffold sequence. (middle) Diagram illustrating the experimental protocol. (bottom) Representative denaturing PAGE gel showing the intrinsic transcript cleavage by the wt- and ΔTL-RNAP as a function of time.

(B) (top) Backtracked EC scaffold (1 nt U mismatch at the 3’-end to yield a 1 nt U backtracked EC) used in the intrinsic cleavage experiments. The RNA cleavage position is shown by an upward arrow below the scaffold sequence. (middle) Diagram illustrating the experimental protocol. (bottom) Representative denaturing PAGE gel showing the intrinsic transcript cleavage by the wt- and ΔTL-RNAP as a function of time.

Fig. S7. Effect of CBR9379 on pyrophosphorolysis and exonucleolytic cleavage on a pre-translocated EC.

(top) Scaffold used in the cleavage experiments (10). The RNA cleavage position is shown by an upward arrow below the scaffold sequence.

(middle) Diagram illustrating the experimental protocol.

(bottom) Representative polyethyleneimine cellulose plate showing the pyrophosphorolysis (yielding pppC*) and exonucleolytic cleavage (yielding pC*) from the starting RNA (RNA10C*).
Supplementary references


Bae et al., Figure S1
Supp. Figure 2

A

Scaffold 5

5’-CCTATAGGATACCTGGGGCT
3’-GGATATCTATGAACCCGCCATCTCCATCTCCATGTGCCCGTATTGGGTTAGGGTTAGCCGACGACCA

AGGAGAGGTACA
CGGCAGATACCACTCCCAATCGGCCTGCTGGT

GGAGAGGUAC

+4 NTPs

*PC (CMP)

- OH

ApC

Transcript extension or Hydrolytic cleavage

Run-off RNA

WT RNAP His₁₀
(on Ni²⁺ beads)

20 min 23 °C 20 min 23 °C EC₁₀ 4 hr ΔTL 23 °C 23 °C pH 8 Time points

RNA: tDNA ntDNA wash [α²³P] CTP wash ±CBR 4 NTPs, 10 μM @ Mg²⁺, 10 mM

EC₁₀

B

<table>
<thead>
<tr>
<th>NTPs (10 μM @)</th>
<th>-</th>
<th>+</th>
<th>+</th>
<th>+</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Br-CBR (μM)</td>
<td>0</td>
<td>0</td>
<td>19</td>
<td>190</td>
<td>1000</td>
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</tbody>
</table>

Run-off →

RNA₁₀C* →

ApC →

*pC →

C

Run-off

100%

100%

100%

0%

0%

0%

[Br-CBR], μM
Bae et al., Figure S3
Bae et al., Figure S4
**Supp. Figure 7**

**Scaffold 5**

\[ \text{AGGAGAGGTACA} \]

\[ 5'\text{-CCTATAGGATACTTGGGGGCT} \]

\[ \text{CGGCGAATACCCATCCCAATCGGCCTGCTGGT} \]

\[ 3'\text{-GGATATCCTATGAAACCACCGATCTCTCTGATGTGCCGCTTTATGGGTTAGGGTTAGCCCGGACGACCA} \]

**Pyrophosphorolysis or Exonuclease cleavage**

**Table:**

<table>
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<th>ΔTL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubation time</td>
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<td>60 min</td>
</tr>
<tr>
<td>Pyrophosphate</td>
<td>–</td>
<td>2 μM</td>
</tr>
<tr>
<td>–</td>
<td>±CBR</td>
<td>±CBR</td>
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<tr>
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</tr>
<tr>
<td>Mg\textsuperscript{2+} (10 mM)</td>
<td>–</td>
<td>+</td>
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<tr>
<td>–</td>
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<td>+</td>
</tr>
<tr>
<td>CBR9379S (μM)</td>
<td>–</td>
<td>100</td>
</tr>
<tr>
<td>–</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>–</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>–</td>
<td>100</td>
<td>10</td>
</tr>
<tr>
<td>–</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>–</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**Diagram:**

- RNA: tDNA
- ntDNA
- wash
- [α\textsuperscript{32P}]CTP
- wash
- ±CBR
- PPI
- Mg\textsuperscript{2+}
- 23 °C
- EC\textsubscript{A9}
- Time points

**Images:**

- pC*
- pppC*
- RNA10C*