Fidaxomicin Is an Inhibitor of the Initiation of Bacterial RNA Synthesis

Irina Artsimovitch,[1] Jaime Seddon,[2] and Pamela Sears[2]

[1]Department of Microbiology, The Ohio State University, Columbus; and [2]Optimer Pharmaceuticals, Inc., San Diego, California

Fidaxomicin was recently approved for the treatment of *Clostridium difficile* infection. It inhibits transcription by bacterial RNA polymerase. Because transcription is a multistep process, experiments were conducted in which fidaxomicin was added at different stages of transcriptional initiation to identify the blocked step. DNA footprinting experiments were also conducted to further elucidate the stage inhibited. Fidaxomicin blocks initiation only if added before the formation of the "open promoter complex," in which the template DNA strands have separated but RNA synthesis has not yet begun. Binding of fidaxomicin precludes the initial separation of DNA strands that is prerequisite to RNA synthesis. These studies show that it has a mechanism distinct from that of elongation inhibitors, such as streptolydigin, and from the transcription initiation inhibitors myxopyronin and the rifamycins.

Fidaxomicin (FDX) was recently approved by the US Food and Drug Administration for the treatment of *Clostridium difficile* infection. FDX is structurally similar to compounds in lipiarmycin (LPR), a fermentation mixture [1, 2], which has been shown to inhibit a variety of bacterial RNA polymerases (RNAPs) [3–7].

Bacterial RNAP is an attractive target for antibiotics. First, RNAP is the central enzyme of gene expression and thus is essential for viability. Second, although the RNAP regions that perform catalysis and establish the key interactions with the nucleic acids are universally conserved from bacteria to humans, eukaryotic nuclear enzymes are insensitive to the inhibitors of bacterial RNAP. Even among bacteria, RNAPs vary greatly [8] because of the constraints imposed by elaborate regulatory networks that adjust the gene expression program to environmental cues; thus, both broad- and narrow-spectrum antibiotics that target RNAP could exist. Third, RNAP performs several enzymatic reactions and interacts with a large number of regulators, providing numerous potential targets for interference by antibiotics. Finally, rifamycins, a class of RNAP inhibitors discovered >50 years ago as fermentation products of *Streptomyces mediterranei* [9], retain their position as first-line antibiotics in combating tuberculosis worldwide.

However, rifamycins remain the only class of RNAP inhibitors in medical practice. Furthermore, their efficiency and versatility are limited by the rapid increase in drug-resistant bacteria, because their contact site on the β-subunit is located in a relatively dispensable region [10, 11]. Antibiotics that differ in their binding sites on the enzyme and the mechanism of inhibition thus are urgently needed. Inhibitors from the FDX family fulfill both criteria.

RESULTS AND DISCUSSION

Establishment of RNAP as a Target of FDX

Because of the similarity in FDX structure to the structures of LPR [1, 2], it was expected that they would share the same target. We found that FDX inhibited both RNAP isolated from *C. difficile* by the method of Pich and Bahl [12] and RNAP from *Escherichia coli* in a radiolabeled uridine triphosphate incorporation

Correspondence: Irina Artsimovitch, Department of Microbiology, The Ohio State University, Columbus, OH 43210 (artsimovitch.1@osu.edu).

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assay [13]. Although the *E. coli* RNAP enzyme is less sensitive than the *C. difficile* RNAP, both enzymes are effectively inhibited by FDX. Mechanistic work was performed with the enzyme from *E. coli*, because this organism is more tractable for mutagenesis and recombinant protein purification.

RNAP Mechanism

During the initiation phase of the transcription cycle (Figure 1*A*), the core RNAP (a complex of an α-dimer, β, β′, and ω subunits) combines with a promoter-specificity factor σ to locate and bind to a promoter region of the DNA, then separates, or melts, the double-stranded DNA to form an open promoter complex (RP_o) in which the melted region (transcription bubble) extends from positions −12 to +2 (relative to the transcription start site). The incoming nucleotide (nucleoside triphosphate) substrate pairs with the template DNA in the enzyme's active site (+1), and RNA synthesis commences. When RNAP adds 1 nucleotide to a growing RNA chain, it moves 1 step forward on the DNA template, repeating this cycle hundreds or thousands of times during the elongation phase until it reaches a terminator.

Order-of-Addition Experiments

Order-of-addition experiments are commonly used to pinpoint a step along the pathway at which an antibiotic acts. In these assays, the antibiotic is added to a free RNAP or to transcription complexes poised at different checkpoints. When the enzyme bypasses the step sensitive to the inhibitor, it becomes resistant to its action. Antibiotics that inhibit RNA chain elongation block transcription when added at any step during transcription; this group includes streptolydigin [14, 15], tagetitoxin [16], microcin J25 [17, 18], and CBRs (a group of synthetic RNA polymerase inhibitors), which are rifamycin-quinolone hybrids [19]. Rifampicin [1] and sorangicin [20] block extension of short transcripts and are no longer able to act when the nascent RNA grows longer than 4 nt. LPR [21] and myxopyronins (MYXs), such as desmethylmyxopyronin (dMYX) [22, 23], inhibit RNAP only if added before formation of the RP_o. We found that FDX also inhibits transcription only if added before the stable RP_o has been formed (Figure 1*B*).

Importantly, a common point of action does not prove the same mechanism. During both initiation and elongation, transcription complexes exist in many interconverting states that are differentially sensitive to both cellular regulators and antibiotics. These states have been particularly well characterized in the course of initiation when several promoter complexes form sequentially, culminating in RP_o [24].

MYX as an Initial Model and Switch-2 Region as a Target

In addition to the similar behavior of FDX, LPR, and dMYX in order-of-addition experiments, the patterns of mutations that confer resistance to FDX, LPR, and dMYX overlap, suggesting a similar site of binding/action on the RNAP. FDX and LPR do not inhibit *Thermus* RNAPs, the only bacterial enzymes for

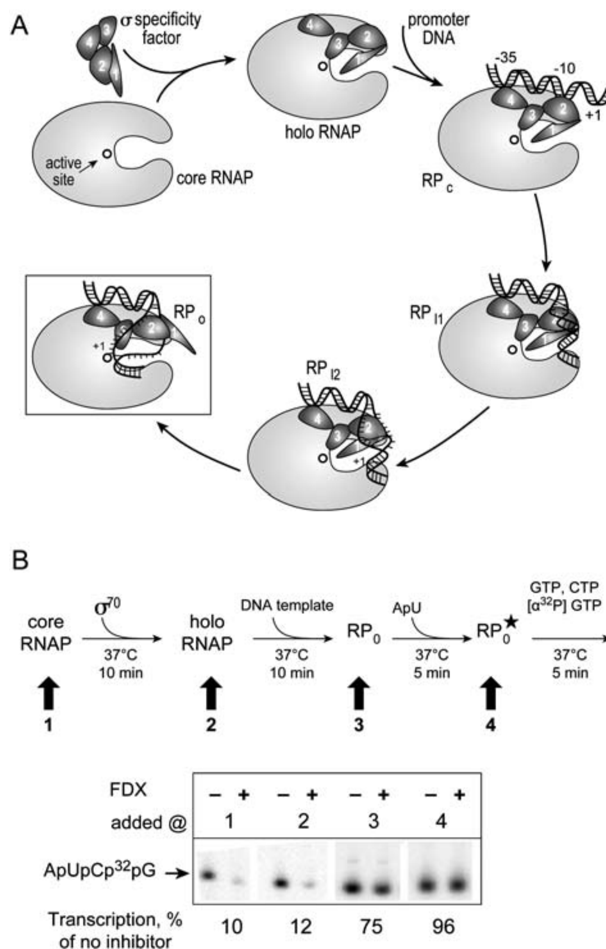

Figure 1. *A*, Transcription initiation pathway. Core RNA polymerase (RNAP; gray oval, with active site depicted as a circle) binds to a promoter specificity σ initiation factor (1 of many) that directs the resulting holo RNAP to a subset of promoters. In *Escherichia coli*, the primary σ^{70} factor consists of separate domains (numbered 1–4); domains 2 and 4 recognize the −10 and −35 elements of "housekeeping" promoters to form a closed promoter complex, RP_c, in which the double-stranded DNA is loosely bound on the surface of RNAP. In RP_{I1}, the first intermediate along the pathway, DNA strand separation initiates around the −11 position (relative to the transcription start site). Melting propagates toward the active site (as shown in RP_{I2}, but additional complexes also may exist). In the final, transcription-competent open promoter complex (RP_o; boxed), the transcription bubble encompasses the +1 position. *B*, Order-of-addition experiments. Fidaxomicin (FDX; at 50 μM) was added to the in vitro transcription reaction with the *E. coli* RNAP at different points. The fraction of synthesized RNA was measured (as percentage of transcription in the absence of the antibiotic). Inhibition of the reaction was observed when FDX was added before steps 1 and 2, but not 3 or 4, after formation of the RP_o. Abbreviations: [α^{32}P]GTP, α-radiolabeled GTP; ApU, Adenylyl (3′-5′) uridine; ApUpC^{32}pG, tetranucleotide reaction product; CTP, cytidine triphosphate; FDX, fidaxomicin; GTP, guanidine triphosphate; $RP_o^{\star}$, open complex stabilized by the addition of a dinucleotide primer ApU.

which detailed structural information currently is available. However, structures of dMYX in complex with *Thermus thermophilus* RNAP revealed that the antibiotic binds to and drastically alters the conformation of a β′ subunit element called switch-2. Structural studies suggested that this element may play 2 important roles in transcription. First, the β′ switch-2 forms a hinge that connects the 2 "pincers" of the RNAP crab-claw-like "clamp" [25]; opening of these pincers is thought to be required to load the DNA into the RNAP during initiation. Second, the β′ switch-2 interacts with the template DNA strand and determines its position. Accordingly, 2 models were proposed to explain inhibition by dMYX. In 1 model, dMYX restricts movements of the β′ switch-2, blocking the clamp opening. In another, dMYX-stabilized refolding of the β′ switch-2 sterically blocks the path of the template DNA strand near the RNAP active site.

Analysis of dMYX-resistant mutants in vivo and in vitro validated the antibiotic-binding site [22, 23]. Footprinting analysis revealed that dMYX does not prevent DNA binding to *E. coli* RNAP or nucleation of DNA melting by the σ subunit. However, dMYX blocks the propagation of DNA melting toward the active site, stabilizing a partially melted intermediate, the existence of which has been postulated for many years [22]. On the basis of the complex structure, we designed substitutions in the β′ switch-2 that mimicked the dMYX-bound conformation even in the absence of the antibiotic; these mutant enzymes were hypersensitive to dMYX and were locked in the same inactive promoter complex intermediate. Of interest, DksA, a regulatory protein in *E. coli*, inhibits transcription through similar changes in RNAP/DNA interactions, and substitutions in the β′ switch-2 alter the enzyme's response to DksA [26].

Switch-2 thus is a popular target for RNAP inhibitors, and LPR-resistant substitutions therein initially suggested that LPR (and based on structural similarities, also FDX) may act similarly to dMYX. For example, the same substitution, β′ R337A, confers resistance both to antibiotics [21, 22] and also to FDX [27]. However, a study by Tupin et al [21] revealed important differences between LPR and dMYX. First, Tupin et al demonstrated that LPR acts at a step preceding the one targeted by dMYX. In the presence of LPR, RNAP bound to the promoter DNA to form an unstable complex, but no strand separation could be detected. Second, this study suggested a key role for the σ subunit in LPR action: deletion of the σ hairpin loop that is positioned near the β′ switch-2 conferred resistance to LPR. Thus, although the binding sites for LPR and dMYX may partially overlap, their mechanisms appear to be distinct. In addition, it remained to be determined whether FDX acted similarly to LPR, particularly because these antibiotics target the highly dynamic switch-2 and their action could be strongly influenced by even subtle differences between the inhibitor scaffolds, the source of RNAP, and the transcription complexes used for the analysis.

Footprinting Analysis to Compare MYX, LPR, and FDX

Footprinting analyses were conducted with FDX to evaluate whether FDX behaved like LPR. Similar to LPR [21] and in contrast to dMYX, FDX inhibited DNA melting in promoter complexes throughout the region surrounding the transcription start site (Figure 2). However, our DNase I footprinting analysis [27] revealed that FDX not only induced changes in the downstream DNA interactions with the RNAP (as do dMYX and LPR), but also altered the DNA conformation in the spacer region of the affected complexes (unlike LPR). Also in contrast to LPR, FDX action was not altered by deletion of the σ hairpin loop. The source of these differences remains to be determined. One possibility is that they are attributable to the different promoters used for these analyses, λ P_R in our work and P_{lacUV5} in Tupin et al [21].

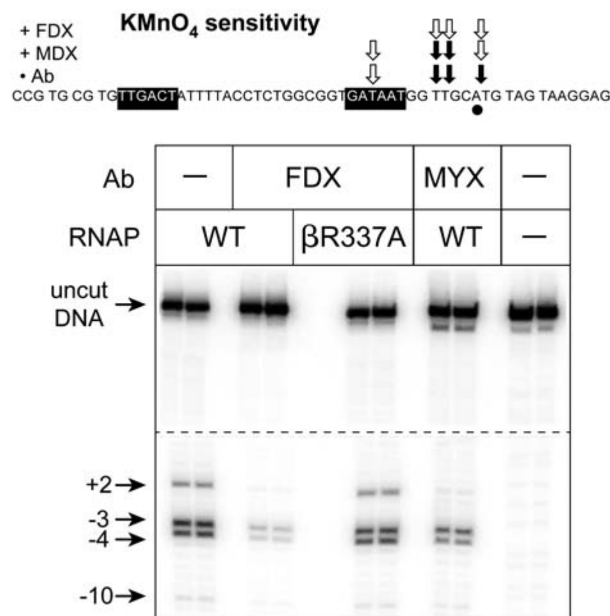

Figure 2. Fidaxomicin (FDX) inhibits DNA strand separation in promoter complexes. Melting of the promoter DNA was probed by single-hit permanganate footprinting. Potassium permanganate ($KMnO_4$) modifies single-stranded thymidine (T) residues, which subsequently are cleaved by piperidine; positions of cleavage indicate the location of single-stranded DNA regions. A linear end-labeled λP_R promoter DNA fragment was generated by polymerase chain reaction. *Top*, The −35 and −10 hexamers are indicated by black boxes, the start site (+1) is shown by a black dot. Reactivities of the −10, −4, −3, and +2 non-template strand T residues to $KMnO_4$ modification are summarized above the sequence; black and white arrows indicate high and low reactivity, respectively. *Bottom*, A representative gel with the sensitive residues shown; the middle portion of the gel was removed (dashed line) to conserve space. Results show that although myxopyronin blocks only DNA melting near the start site (at +2 position), FDX reduces strand separation throughout the entire sensitive region with the wild-type RNAP, but not with a mutant that carries an R337A substitution in the β′ switch-2 region. Abbreviations: Ab, antibiotic; FDX, fidaxomicin; MYX, myxopyronin; RNAP, RNA polymerase; WT, wild-type.

RNAP presents a complex target for inhibition, and changes in the enzyme or antibiotic may confer substantial differences. Small changes in antibiotic structure may alter the contacts with the enzyme and consequently change the point for inhibition, as illustrated by the case of rifamycins [10]. Substitutions in RNAP (naturally occurring in different bacterial enzymes) bestow distinct kinetic properties and altered sensitivity to cellular effectors and antibiotics alike. Some of these changes impair the binding of a ligand to RNAP, but others may act indirectly by altering the enzyme's conformation and changing its response to the antibiotic. For example, a subset of CBR-resistant mutations in *rpoC* conferred dependence on the inhibitor for growth of the mutant strains [19], indicating that their phenotypes cannot be explained by the loss of CBR binding. Thus, the phenotypes of any resistance mutations should be interpreted with caution because some of them could have indirect (allosteric) effects. This may be particularly common for antibiotics that bind to the key sites on RNAP, where substitutions would give rise to growth defects. This complexity demands that when possible, structural analysis of the antibiotic/transcription complexes should be pursued in parallel with in-depth functional and genetic studies, using target RNAPs from relevant bacterial species. Nevertheless, functional studies are useful for identifying differences in mechanism, particularly because they can provide information about the dynamics of enzyme inhibition rather than focusing on a static picture of binding. The mechanistic studies presented here confirm that there are multiple steps that are targets for RNAP inhibition by different antibiotics, and FDX inhibits a step distinct from elongation inhibitors, such as streptolydigin, and from the initiation inhibitors MYX and the rifamycins. The difference in mechanism compared with the rifamycins supports previous findings of lack of cross-resistance with this class of antibiotics [28] and suggests that the rapid development of resistance observed with rifamycins does not indicate a rapid loss of susceptibility to FDX.

Notes

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