

Fluoroquinolone interactions with *Mycobacterium tuberculosis* gyrase: Enhancing drug activity against wild-type and resistant gyrase

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Mycobacterium tuberculosis is a significant source of global morbidity and mortality. Moxifloxacin and other fluoroquinolones are important therapeutic agents for the treatment of tuberculosis, particularly multidrug-resistant infections. To guide the development of new quinolone-based agents, it is critical to understand the basis of drug action against *M. tuberculosis* gyrase and how mutations in the enzyme cause resistance. Therefore, we characterized interactions of fluoroquinolones and related drugs with WT gyrase and enzymes carrying mutations at GyrA^{A90} and GyrA^{D94}. *M. tuberculosis* gyrase lacks a conserved serine that anchors a water–metal ion bridge that is critical for quinolone interactions with other bacterial type II topoisomerases. Despite the fact that the serine is replaced by an alanine (i.e., GyrA^{A90}) in *M. tuberculosis* gyrase, the bridge still forms and plays a functional role in mediating quinolone–gyrase interactions. Clinically relevant mutations at GyrA^{A90} and GyrA^{D94} cause quinolone resistance by disrupting the bridge–enzyme interaction, thereby decreasing drug affinity. Fluoroquinolone activity against WT and resistant enzymes is enhanced by the introduction of specific groups at the C7 and C8 positions. By dissecting fluoroquinolone–enzyme interactions, we determined that an 8-methyl-moxifloxacin derivative induces high levels of stable cleavage complexes with WT gyrase and two common resistant enzymes, GyrA^{A90V} and GyrA^{D94G}. 8-Methyl-moxifloxacin was more potent than moxifloxacin against WT *M. tuberculosis* gyrase and displayed higher activity against the mutant enzymes than moxifloxacin did against WT gyrase. This chemical biology approach to defining drug–enzyme interactions has the potential to identify novel drugs with improved activity against tuberculosis.

Mycobacterium tuberculosis | fluoroquinolones | antibiotic resistance | gyrase | complex stability

Tuberculosis is a major cause of morbidity and mortality on a global scale and is second only to HIV/AIDS as the most prolific killer among single infectious agents (1). According to the World Health Organization, 9 million people were diagnosed with tuberculosis in 2013, and 1.5 million died from the disease (1). The standard treatment regimen for tuberculosis is a 6-month course that includes a combination of rifampin, isoniazid, pyrazinamide, and ethambutol (2, 3). However, fluoroquinolones are becoming more important in the treatment of tuberculosis and are routinely used in multidrug-resistant cases and in patients who are intolerant of first-line therapy (2, 4, 5). Furthermore, moxifloxacin (a newer-generation fluoroquinolone) has shown promising results as a potential first-line agent as part of the PaMZ regimen (PA-824, moxifloxacin, and pyrazinamide), which currently is in clinical trials (6).

Fluoroquinolones are broad-spectrum antibacterial agents that act by increasing levels of DNA strand breaks generated by type II topoisomerases (7–12). Most bacterial species encode two type II enzymes, gyrase and topoisomerase IV (8, 10, 12–14). In these species, gyrase regulates the superhelical density of the bacterial chromosome and removes torsional stress that is gen-

erated ahead of DNA replication forks and transcription complexes, and topoisomerase IV primarily unknots and untangles DNA (11, 15, 16). *Mycobacterium tuberculosis*, the causative agent of tuberculosis, is unusual in that it encodes only gyrase (17). As a result, this enzyme displays functional properties of both type II topoisomerases (18).

Recent structural (19) and functional (20, 21) studies with topoisomerase IV indicate that quinolones interact with bacterial type II enzymes primarily through a water–metal ion bridge. This bridge is formed by a divalent metal ion that is chelated by the C3/C4 keto acid of the drug and stabilized by four water molecules (19). Two of these water molecules are coordinated by a conserved serine and acidic residue (located four positions downstream) in the A subunit of the enzyme. Substitutions in the residues that anchor the bridge are the most prevalent cause of quinolone resistance (10–13, 22–27). In most species, the serine is mutated far more often than the acidic residue.

In contrast to most bacterial species, *M. tuberculosis* gyrase contains an alanine (A90) in place of the conserved serine. This situation raises the issue of whether the water–metal ion bridge can be formed or plays a role in mediating quinolone activity in this species. However, the fact that mutations at A90 and

Significance

Moxifloxacin and other fluoroquinolone antibacterial agents are important antituberculosis therapeutic agents. Fluoroquinolones kill *Mycobacterium tuberculosis*, the causative agent of tuberculosis, by increasing levels of DNA breaks generated by gyrase, an essential type II topoisomerase that regulates DNA topology. As fluoroquinolone use in antituberculosis regimens is becoming more pronounced, understanding the basis of drug–gyrase interactions and resistance is becoming more important. By using a mechanism-based chemical biology approach, our work identified critical drug features that mediate fluoroquinolone interactions with *M. tuberculosis* gyrase and determined the biochemical basis for fluoroquinolone resistance caused by the most common clinical mutations in gyrase. These findings allowed us to identify a moxifloxacin derivative that displays enhanced activity against WT gyrase and maintains high activity against clinically relevant resistant enzymes.

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the acidic residue (D94) are associated with clinical quinolone resistance in *M. tuberculosis* (28) suggests that the bridge contributes to quinolone function.

Fluoroquinolones are commonly prescribed for community-acquired pneumonia that is later diagnosed as pulmonary tuberculosis (29). This prior treatment is associated with an increased incidence of fluoroquinolone-resistant disease (30, 31). As the use of fluoroquinolones in treating tuberculosis is becoming more pronounced, understanding the basis of drug–gyrase interactions and resistance is becoming more important. Therefore, we analyzed the interactions of fluoroquinolones and related compounds with WT and resistant mutant *M. tuberculosis* gyrase. Results indicate that the water–metal ion bridge is partially functional in WT gyrase and that the most common resistance mutations cause a decrease in bridge-mediated drug affinity for the enzyme. In contrast to other species (32, 33), quinolone interactions within the gyrase-cleaved DNA complex depend more heavily on substituents at C7 and C8. Based on an analysis of structure–activity relationships at these two positions, we identified fluoroquinolones that display significantly improved activity against WT and resistant *M. tuberculosis* gyrase compared with moxifloxacin.

Results and Discussion

Gyrase is a heterotetramer comprised of two subunits, GyrA (which contains the active site tyrosine that cleaves and ligates DNA) and GyrB (which contains the ATPase and metal-binding domains) (8, 10, 12–14). To characterize interactions between quinolones and *M. tuberculosis* gyrase, we used WT enzyme and GyrA^{A90S}, GyrA^{A90V}, GyrA^{D94G}, and GyrA^{D94H}. Amino acid residues A90 and D94 occur at the positions that, by sequence homology, are predicted to anchor the water–metal ion bridge if it is used to mediate quinolone–enzyme interactions in this species (19–21). The GyrA^{A90V}, GyrA^{D94G}, and GyrA^{D94H} proteins contain three of the most common mutations associated with quinolone resistance in clinical isolates of *M. tuberculosis* (28). The GyrA^{A90S} protein replaces the alanine found in the WT enzyme with a serine, which is the residue that serves as a bridge anchor in most bacterial type II topoisomerases. The positions of GyrA^{A90} and GyrA^{D94} relative to bound quinolones are described in the accompanying paper by Blower et al. (34).

The GyrB subunit of *M. tuberculosis* gyrase was reannotated based on sequence alignments with 50 other bacterial species (35). It is now believed that the start codon is GTG (rather than ATG), which results in a protein that is 675 aa in length, rather than 714 aa (28). The experiments described in the present paper used GyrB that reflects this updated annotation.

Enzymatic Activities of WT and Quinolone-Resistant Mutant *M. tuberculosis* Gyrase. Before analyzing fluoroquinolone action against the WT and GyrA^{A90S}, GyrA^{A90V}, GyrA^{D94G}, and GyrA^{D94H} mutant *M. tuberculosis* gyrase proteins, we determined baseline DNA supercoiling and cleavage activities for the enzymes in the absence of drugs (Fig. 1). Each mutant enzyme maintained high DNA supercoiling activity and, in the presence of Ca²⁺ (used to increase baseline levels of enzyme-mediated DNA scission), cleaved DNA at least as well as WT gyrase. Thus, quinolone resistance does not correlate with a loss of baseline activity for any of the mutant enzymes examined.

Effects of Fluoroquinolones and Related Compounds on DNA Cleavage Mediated by WT and Fluoroquinolone-Resistant Mutant *M. tuberculosis* Gyrase. The first set of experiments compared the ability of ciprofloxacin and moxifloxacin to induce DNA cleavage by WT gyrase and the mutant enzymes (Fig. 2, *Top*). The two drugs generated similar levels of cleavage with the WT enzyme. In contrast, moxifloxacin maintained higher activity against the GyrA^{A90V}, GyrA^{D94G}, and GyrA^{D94H} mutant proteins. As shown previously, the D94G and D94H mutations caused a higher level of fluoroquinolone resistance than the A90V mutation (36, 37).

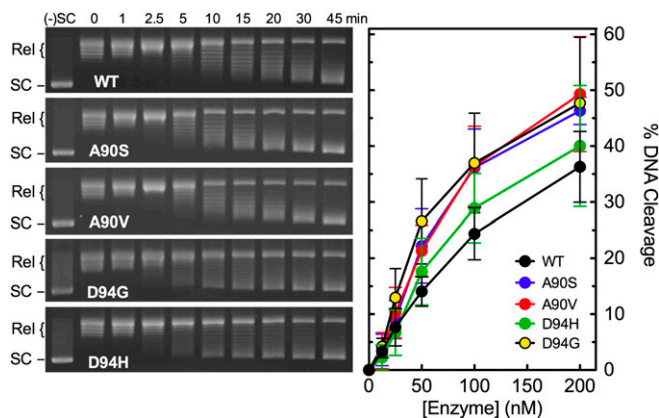


Fig. 1. Catalytic and DNA cleavage activities of WT and mutant *M. tuberculosis* gyrase in the absence of drugs. (*Left*) The ability of WT (black), GyrA^{A90S} (A90S, blue), GyrA^{A90V} (A90V, red), GyrA^{D94H} (D94H, green), and GyrA^{D94G} (D94G, yellow) to supercoil relaxed pBR322 plasmid DNA is shown. Gels are representative of three independent experiments. The positions of negatively supercoiled [(-)SC] and relaxed (Rel) DNA controls are indicated. (*Right*) The ability of the enzymes to cleave negatively supercoiled pBR322 plasmid DNA is shown. Error bars represent the SD of at least three independent experiments.

The aforementioned findings are consistent with the hypothesis that fluoroquinolones interact with WT *M. tuberculosis* gyrase through a water–metal ion bridge that is anchored primarily by D94. To investigate this possibility, the activity of ciprofloxacin and moxifloxacin against GyrA^{A90S} was examined. The inclusion of the serine residue in place of the alanine provides the potential to reconstitute a fully functional bridge. As seen in Fig. 2 (*Top*), both fluoroquinolones displayed dramatically higher activity against GyrA^{A90S} than they did against WT gyrase: drug potency (i.e., drug concentration required to induce 50% maximal DNA cleavage) was approximately fivefold higher, and levels of cleavage at 100 μ M were approximately twofold higher. Collectively, these findings suggest that there is sufficient conservation of structure such that *M. tuberculosis* gyrase is capable of using a water–metal ion bridge to mediate fluoroquinolone–enzyme interactions. This conclusion is supported by the structural studies in the accompanying paper by Blower et al. (34).

Quinazolinodiones are fluoroquinolone-like compounds that lack the C3/C4 keto acid required to chelate metal ions. In several other species, quinazolinodiones containing a 3'-(aminomethyl) pyrrolidinyl [3'-(AM)P] (or a related) group at C7 maintain activity against enzymes containing mutations that disrupt the water–metal ion bridge by forming binding interactions primarily through the C7 substituent (20, 21, 32, 38–42). As seen in Fig. 2 (*Middle Left*), the activity of 8-methyl-3'-(AM)P-dione is unaffected by mutations that could disrupt or strengthen the water–metal ion bridge in *M. tuberculosis* gyrase. The activity of 8-methyl-3'-(AM)P-FQ (the fluoroquinolone version of this quinazolinodione) is unaffected by bridge-disrupting mutations but is enhanced upon the introduction of the serine residue at position 90 (Fig. 2, *Middle Right*). Again, these findings support the hypothesis that clinically relevant fluoroquinolones interact with *M. tuberculosis* gyrase through the water–metal ion bridge and that D94 likely mediates a partial bridging interaction in the absence of the upstream serine.

Fluoroquinolones that have a C8 substituent generally display better activity against clinical tuberculosis isolates than do derivatives with a C8-H (43, 44). Therefore, 3'-(AM)P-quinazolinodione and -fluoroquinolone derivatives that contained a C8-H were used to examine the influence of the C8 substituent on drug activity (Fig. 2, *Bottom*). The activity of 8-H-3'-(AM)P-dione was

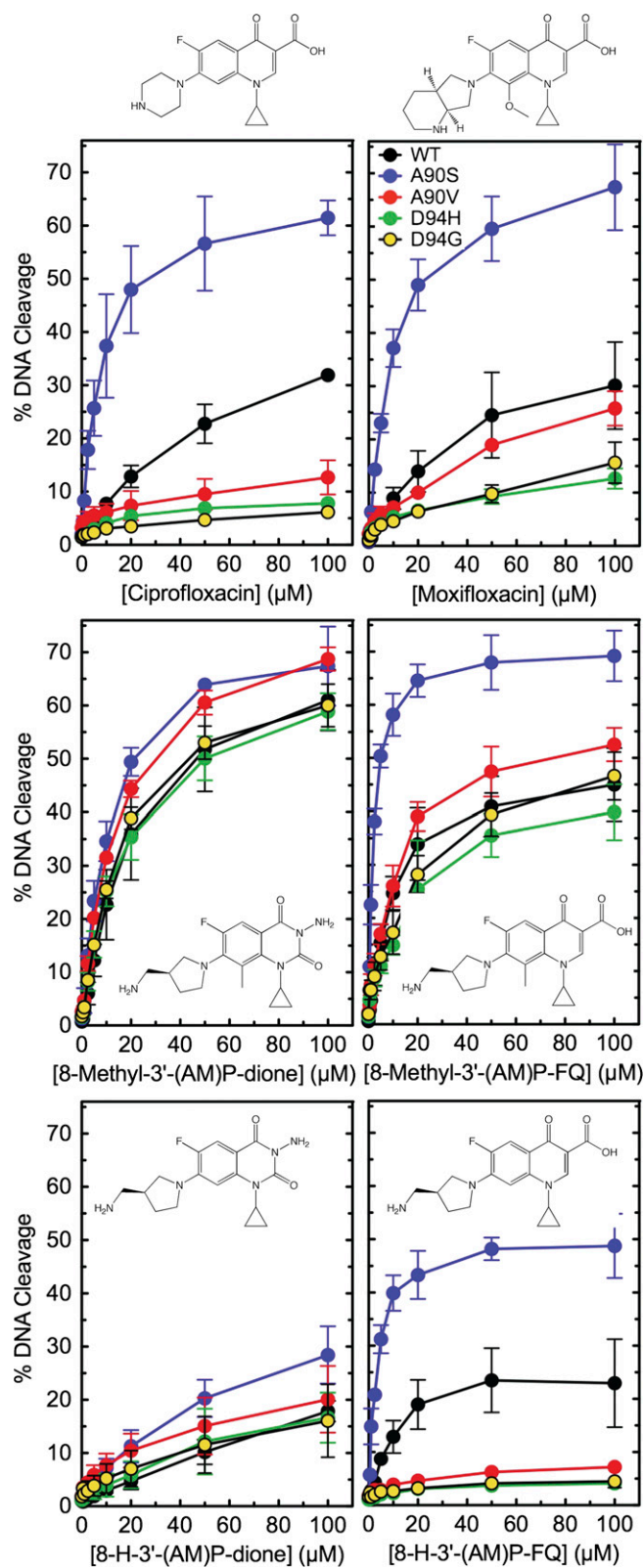


Fig. 2. Drug-induced DNA cleavage mediated by WT and mutant *M. tuberculosis* gyrase. The ability of WT (black), GyrA^{A90S} (A90S, blue), GyrA^{A90V} (A90V, red), GyrA^{D94H} (D94H, green), and GyrA^{D94G} (D94G, yellow) to mediate DNA cleavage in the presence of the clinically used fluoroquinolones ciprofloxacin and moxifloxacin (Top Left and Top Right, respectively), the experimental quinazolidinones 8-methyl-3'-(AM)P-dione and 8-H-3'-(AM)P-dione (Middle Left and Bottom Left, respectively), and the experimental fluoroquinolones 8-methyl-3'-(AM)P-FQ and 8-H-3'-(AM)P-FQ (Middle Right

markedly decreased against all of the enzymes, confirming that the C8 methyl group plays an important role in mediating the activity of these compounds. The activity of 8-H-3'-(AM)P-FQ also decreased with each enzyme compared with the C8-methyl version of this fluoroquinolone. The relative activity of 8-H-3'-(AM)P-FQ correlated with the capacity of each enzyme to anchor the water–metal ion bridge.

Taken together, these findings strongly suggest that WT *M. tuberculosis* gyrase partially supports the water–metal ion bridge and that the bridge and the C7 and C8 substituents play important roles in supporting quinolone activity. Therefore, the contributions of these three drug features to quinolone interactions and activity were analyzed in greater detail.

Role of the Water–Metal Ion Bridge in Facilitating Quinolone Activity Against *M. tuberculosis* Gyrase. To determine whether quinolone interactions with *M. tuberculosis* gyrase are mediated by the water–metal ion bridge, Mg²⁺ titrations were carried out to assess the effects of A90 mutations on the metal ion concentration required to support DNA cleavage induced by ciprofloxacin (Fig. 3, Left). The amino acid at this position plays a critical role in anchoring the water–metal ion bridge in other bacterial type II enzymes (20, 21, 33). Results with WT gyrase were compared with those of mutant enzymes containing a serine or a valine in place of the alanine at position 90. The GyrA^{A90S} mutant enzyme, which contains the conserved serine residue found in most other bacterial type II enzymes and greatly enhances fluoroquinolone activity (see Fig. 2), required a lower concentration of Mg²⁺ to achieve half-maximal and maximal levels of quinolone-induced DNA cleavage. In marked contrast, the GyrA^{A90V} mutant enzyme, which causes quinolone resistance (see Fig. 2), required a higher concentration of Mg²⁺ to achieve half-maximal and maximal levels of quinolone-induced DNA cleavage.

These results demonstrate that the amino acid at position 90 affects the affinity of the divalent metal ion that is chelated by the quinolone and indicate that the water–metal ion bridge is a point of contact between quinolones and *M. tuberculosis* gyrase. It is likely that the alanine at position 90 in WT gyrase, although it does not serve as a bridge anchor, still allows D94 to anchor the bridge. However, because *M. tuberculosis* gyrase has only one available amino acid to anchor the bridge, fluoroquinolone interactions are weaker than seen with species that encode two amino acid bridge anchors (20, 21, 33). Substitution of A90 with the larger valine may cause quinolone resistance by interfering with the ability of water molecules to coordinate properly with the Mg²⁺ or by impeding D94 interactions with the bridge.

As a control for the aforementioned experiment, the effects of A90 mutations on the Mg²⁺ requirement for DNA cleavage induced by 8-methyl-3'-(AM)P-dione (which does not require a metal ion for enzyme interactions) were determined. In contrast to results with ciprofloxacin, similar levels of Mg²⁺ were required to achieve half-maximal and maximal cleavage with all three enzymes (Fig. 3, Right).

In other bacterial type II topoisomerases, mutations in bridge-anchoring residues that partially disrupt the water–metal ion bridge often restrict the variety of metal ions that can be used to form the bridge (21, 33). Therefore, we examined the ability of a second divalent metal ion, Mn²⁺, to support ciprofloxacin activity against WT gyrase and enzymes with mutations at position 90 (Fig. 4). With GyrA^{A90S}, which has a fully intact bridge, the level of ciprofloxacin-induced DNA cleavage was unaffected by the substitution of this metal ion for Mg²⁺. However, with WT gyrase, which has a partially disrupted bridge, ciprofloxacin activity

and Bottom Right, respectively) is shown. Compound structures are shown in or above their respective panels. Error bars represent the SD of at least three independent experiments.

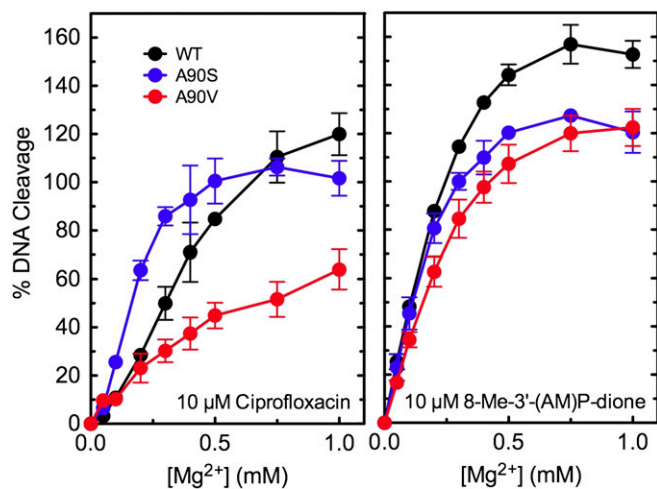


Fig. 3. Effects of Mg^{2+} concentration on DNA cleavage mediated by WT and mutant *M. tuberculosis* gyrase. Results are shown for 10 μ M ciprofloxacin (Left) and 10 μ M 8-methyl-3'-(AM)P-dione (Right) with WT (black), GyrA^{A90S} (A90S, blue), and GyrA^{A90V} (A90V, red). DNA cleavage for each drug–enzyme pair was normalized to 100% at 6 mM Mg^{2+} to facilitate direct comparisons. Error bars represent the SD of at least three independent experiments.

was decreased approximately twofold. Furthermore, with GyrA^{A90V}, which has a more severely disrupted bridge, the quinolone displayed no ability to enhance enzyme-mediated DNA cleavage in the presence of Mn^{2+} . Therefore, relative levels of resistance seen with enzymes containing mutations in bridge-anchoring residues can be altered by the metal ion used to support this interaction. A stronger water–metal ion bridge allows more latitude with metal ion requirements.

The water–metal ion bridge has been shown to play critical roles in mediating fluoroquinolone binding and positioning, but its predominant role appears to differ across species. With *Bacillus anthracis* topoisomerase IV, it primarily acts as a binding contact (20, 21). However, with *Escherichia coli* topoisomerase IV, it has little effect on drug affinity and is believed to properly position the drug in a manner that promotes DNA cleavage (33). Therefore, to determine whether the water–metal ion bridge plays a major role in drug–enzyme binding in *M. tuberculosis* gyrase, we used a competition approach and examined the effects of ciprofloxacin on DNA cleavage induced by 10 μ M 8-methyl-3'-(AM)P-dione in two enzymes with impaired bridge function (GyrA^{A90V} and GyrA^{D94G}). In an enzyme with optimal bridge function (GyrA^{A90S}), the fluoroquinolone and quinazolinone display similar abilities to induce gyrase-mediated DNA cleavage and act with comparable potencies (see Fig. 2, Left, Top, and Middle).

With both resistant mutant enzymes (GyrA^{A90V} and GyrA^{D94G}), ciprofloxacin competed poorly against the bridge-independent quinazolinone (Fig. 5). Even at a 10-fold molar excess of the quinolone, levels of DNA cleavage decreased by only ~25%. This finding implies that impaired bridge function decreases the affinity of ciprofloxacin for the enzyme by at least an order of magnitude and strongly suggests that, in *M. tuberculosis* gyrase, the water–metal ion bridge plays a critical role in mediating quinolone binding.

Enhancing Fluoroquinolone Activity Against WT and Resistant *M. tuberculosis* Gyrase by Modifying Substituents at C7 and C8.

Previous studies (43, 44) have shown that fluoroquinolones containing a substituent at C8 (in particular, a methoxy group) display higher activity against *M. tuberculosis* and related organisms. However, the basis for the effects of C8 substitutions on fluoroquinolone activity against *M. tuberculosis* gyrase is not well understood. Therefore, we compared the activities

of fluoroquinolones containing a C8-H, -methyl, or -methoxy group against the WT and resistant enzymes. Two parallel series were examined: one that contained the C7 piperazinyl ring of ciprofloxacin, and one that contained the C7 diazabicyclononyl ring of moxifloxacin.

The first set of experiments examined drug effects against WT gyrase. In general, the inclusion of a C8 substituent enhanced quinolone activity (Fig. 6, Top Left). For the ciprofloxacin-based series, the compound with a C8 methoxy group generated the highest level of DNA cleavage activity. Compared with ciprofloxacin, 8-methoxy-ciprofloxacin (8-methoxy-cipro) induced ~50% more cleavage at 100 μ M drug and displayed a potency that was ~twofold higher (based on the drug concentration required to induce 50% of the cleavage generated at 100 μ M drug). The presence of a C8 methyl group enhanced DNA cleavage, but to a lesser extent than did the methoxy group.

A more dramatic effect was seen when the C8 methoxy group of moxifloxacin was replaced with a methyl group. Compared with moxifloxacin (the fluoroquinolone most commonly used to treat tuberculosis) (2, 4–6), 8-methyl-moxifloxacin (8-methyl-moxi) induced approximately twofold more cleavage at 100 μ M drug and was approximately twofold more potent. Furthermore, only 10 μ M 8-methyl-moxi was required to induce the same level of scission (~30%) as was seen with 100 μ M moxifloxacin. In contrast, 8-H-moxifloxacin (8-H-moxi) displayed an activity similar to that of moxifloxacin.

The second set of experiments examined the effects of the C8 substituent on fluoroquinolone activity against the resistant GyrA^{A90V} and GyrA^{D94G} mutant enzymes (Fig. 6, Top Right and Bottom Left, respectively). When the function of the water–metal ion bridge was disrupted, the C8 substituent had a much greater influence on drug activity. Drugs lacking a C8 substituent (ciprofloxacin and 8-H-moxi) showed little activity against either fluoroquinolone-resistant enzyme. However, in the presence of a C8 methyl or methoxy substituent, high levels of activity were maintained. To this point, 8-methyl-moxi displayed nearly WT activity against GyrA^{A90V}. Moreover, even though its activity against GyrA^{D94G} was decreased, 8-methyl-moxi still induced higher levels of cleavage than did moxifloxacin with WT gyrase. Representative gels showing the effects of moxifloxacin and

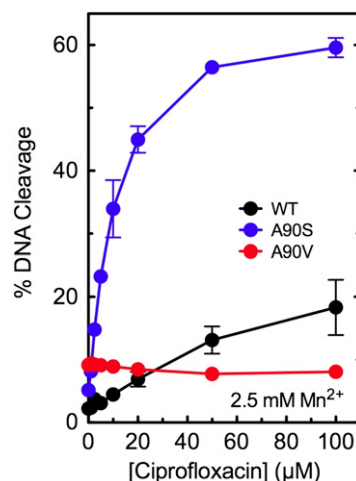


Fig. 4. Effects of Mn^{2+} on drug-induced DNA cleavage mediated by WT and mutant *M. tuberculosis* gyrase. Results are shown for cleavage mediated by WT (black), GyrA^{A90S} (A90S, blue), and GyrA^{A90V} (A90V, red) in the presence of ciprofloxacin. Assays included 2.5 mM Mn^{2+} , the concentration that yielded maximal enzyme activity, instead of Mg^{2+} . Error bars represent the SD of at least three independent experiments.

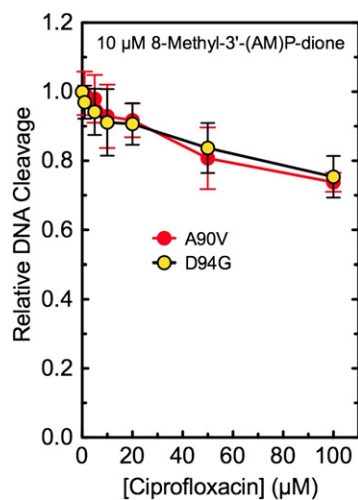


Fig. 5. Ability of ciprofloxacin to compete out DNA cleavage induced by 10 μM 8-methyl-3'-(AM)P-dione with mutant *M. tuberculosis* gyrase. Results are shown for GyrA^{A90V} (A90V, red) and GyrA^{D94G} (D94G, yellow). Both drugs were added to reaction mixtures simultaneously. The low level of cleavage seen in the presence of ciprofloxacin alone was used as a baseline and was subtracted from DNA scission observed in the presence of both drugs. The level of DNA cleavage observed in the presence of the quinazolidinone alone was set to 1.0 to facilitate direct comparisons. Error bars represent the SD of at least three independent experiments.

8-methyl-moxi on DNA cleavage mediated by the mutant GyrA^{D94G} enzyme are shown in Fig. S1.

Some fluoroquinolones and related compounds that display high activity against resistant bacterial type II topoisomerases cross-react with human topoisomerase II α , excluding them from clinical development as antibacterial drugs. For example, CP-115,955 induces higher levels of cleavage with the human enzyme than does etoposide, a commonly prescribed anticancer drug (45, 46) (Fig. 6, *Bottom Right*). Notably, none of the ciprofloxacin- or moxifloxacin-based compounds used in this study displayed significant activity against human topoisomerase II α .

To further understand the contributions of the C7 and C8 substituents to quinolone activity, we determined the abilities of the two least effective compounds (ciprofloxacin and 8-H-moxi) to compete with the two most effective drugs (8-methoxy-cipro and 8-methyl-moxi; Fig. 7, *Left* and *Right*, respectively). The GyrA^{A90V} and GyrA^{D94G} mutant enzymes were used so that the contributions of the C7 and C8 groups would not be obscured by the water-metal ion bridge. A representative gel showing the effects of ciprofloxacin and 8-H-moxi on DNA cleavage mediated by the GyrA^{D94G} mutant gyrase in the presence of 50 μM 8-methoxy-cipro is shown in Fig. S2. Three observations were noted. First, the concentrations of ciprofloxacin and 8-H-moxi required to compete out 50% of the DNA cleavage induced by 50 μM 8-methyl-cipro or 8-methyl-moxi were 100 μM or higher. This finding (which is consistent with the potency data discussed for Fig. 6) indicates that the C8-H derivatives display a decreased affinity for the enzymes compared with 8-methoxy-cipro and 8-methyl-moxi. Thus, a C8 group appears to contribute to drug binding within the enzyme-DNA cleavage complex. Second, compounds bearing a C7 diazabicyclononyl group (as found in moxifloxacin) competed better than those with a C7 piperazinyl substituent (as found in ciprofloxacin), suggesting that moxifloxacin-based, as opposed to ciprofloxacin-based, analogs interact more tightly with *M. tuberculosis* gyrase. Third, each compound competed less effectively against 8-methyl-moxi than against 8-methoxy-cipro, indicating that 8-methyl-moxi interacts more tightly with the enzyme. This finding is consistent with the ob-

servation that 8-methyl-moxi induced higher levels of DNA cleavage with all enzymes tested than did the other fluoroquinolones in these two series (see Fig. 6).

Finally, to further examine the contributions of the C7 and C8 substituents to quinolone action, we determined the persistence of cleavage complexes formed in the presence of ciprofloxacin, moxifloxacin, 8-methoxy-cipro, and 8-methyl-moxi (Fig. 8). Moxifloxacin induced more stable cleavage complexes than ciprofloxacin with all three enzymes. This finding is consistent with binding studies (47)

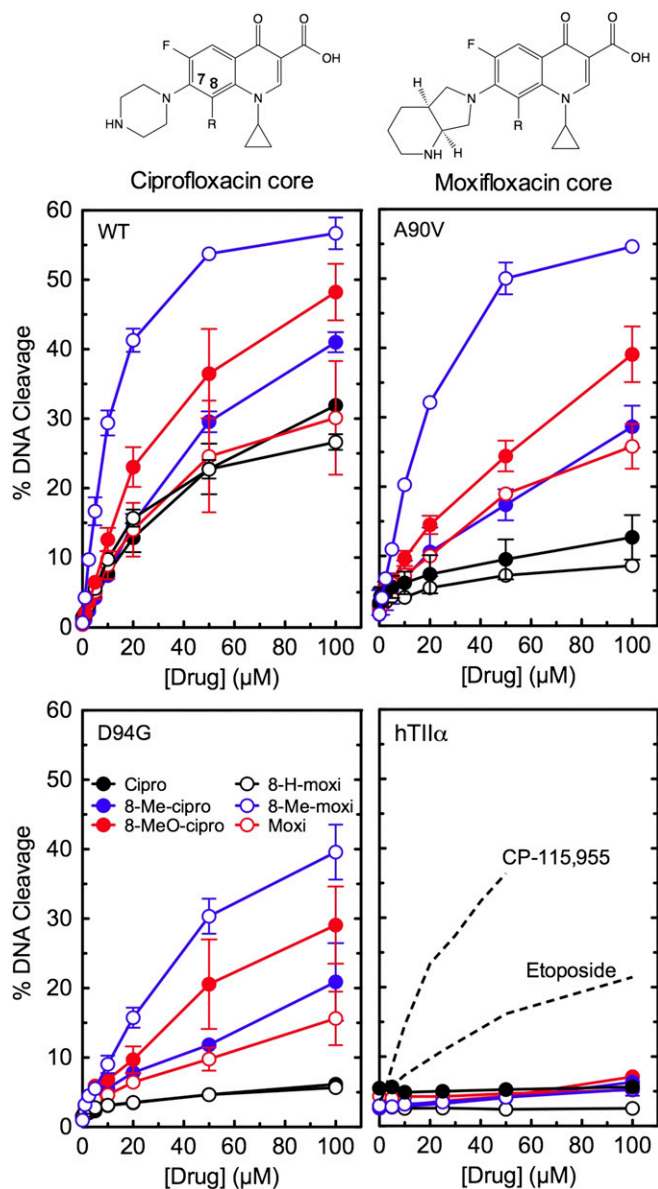


Fig. 6. Effects of ciprofloxacin- and moxifloxacin-based fluoroquinolones on DNA cleavage mediated by WT and mutant *M. tuberculosis* gyrase and human topoisomerase II α . The ability of WT (*Top Left*), GyrA^{A90V} (A90V, *Top Right*), and GyrA^{D94G} (D94G, *Bottom Left*) gyrase and human topoisomerase II α (hTII α , *Bottom Right*) to cleave DNA in the presence of fluoroquinolones containing a C8-H (black), -methyl (blue), or -methoxy (red) group and a C7 piperazinyl (closed symbols) or diazabicyclononyl (open symbols) group is shown. For human topoisomerase II α , DNA cleavage levels induced by the enzyme in the presence of CP-115,955 or etoposide (dashed lines) are shown for comparison. These latter data are from Aldred et al. (32). The ciprofloxacin and moxifloxacin fluoroquinolone cores are shown at the top. Error bars represent the SD of at least three independent experiments.

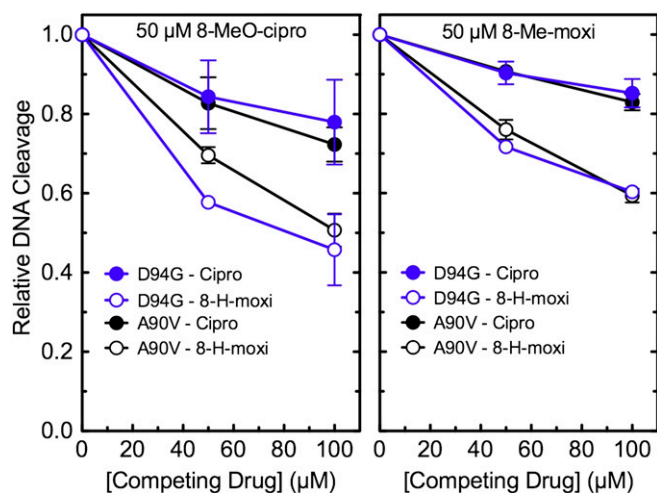


Fig. 7. Ability of fluoroquinolones with a C8-H to compete out DNA cleavage induced by 50 μM 8-methoxy-cipro or 8-methyl-moxi with mutant *M. tuberculosis* gyrase. Results are shown for GyrA^{A90V} (A90V, black) and GyrA^{D94G} (D94G, blue). Ciprofloxacin (Cipro, closed symbols) or 8-H-moxi (open symbols) and 8-methoxy-cipro (*Left*) or 8-methyl-moxi (*Right*) were added to reaction mixtures simultaneously. The low level of cleavage seen in the presence of the C8-H fluoroquinolones alone was used as a baseline and was subtracted from the DNA scission observed in the presence of both drugs. The level of DNA cleavage observed in the presence of 8-methoxy-cipro or 8-methyl-moxi alone was set to 1.0 to facilitate direct comparisons. Error bars represent the SD of at least three independent experiments.

suggesting that moxifloxacin binds more tightly than ciprofloxacin to *M. tuberculosis* gyrase. Furthermore, consistent with the competition experiments, compounds containing the C7 diazabicyclononyl group of moxifloxacin induced cleavage complexes that were more stable than those formed with the C7 piperazinyl group of ciprofloxacin

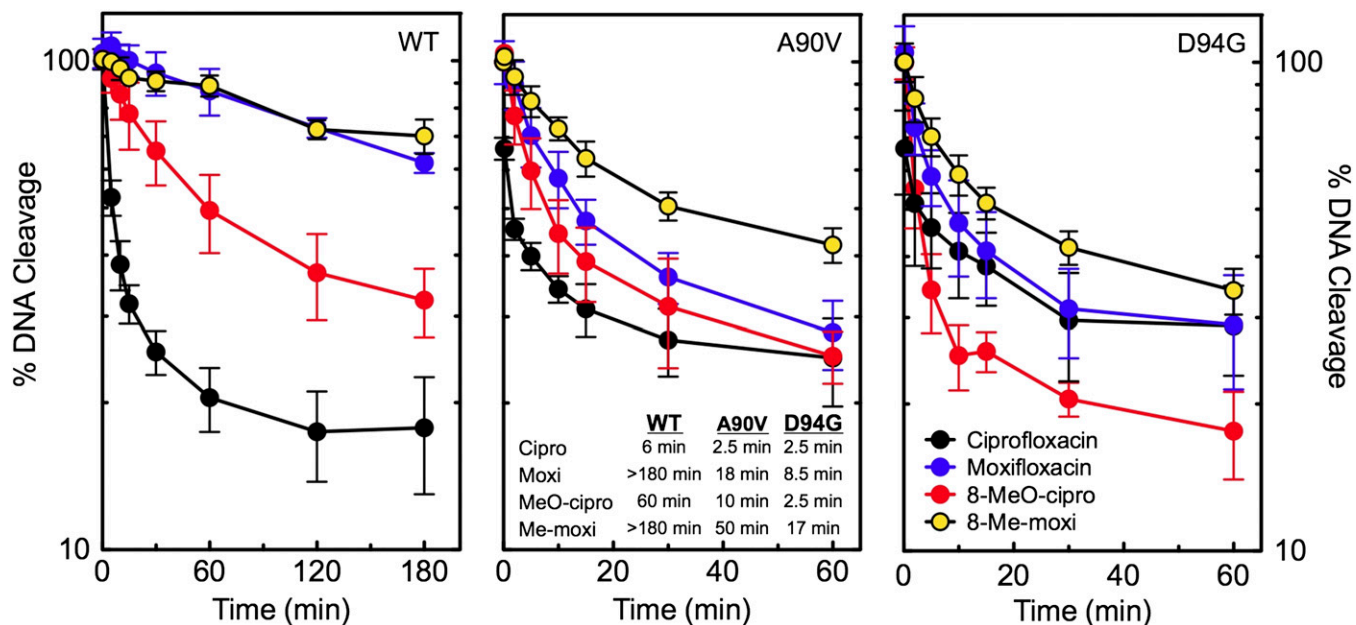


Fig. 8. Effects of fluoroquinolones on the persistence of cleavage complexes formed by WT and mutant *M. tuberculosis* gyrase. The stability of ternary enzyme–drug–DNA cleavage complexes formed with WT (*Left*), GyrA^{A90V} (A90V, *Middle*), and GyrA^{D94G} (D94G, *Right*) was determined in the presence of 100 μM ciprofloxacin (Cipro, black), 50 μM moxifloxacin (Moxi, blue), 50 μM 8-methoxy-cipro (red), or 10 μM 8-methyl-moxi (yellow). The data table (*Inset, Middle*) lists the $t_{1/2}$ of DNA cleavage complexes formed with each drug–enzyme combination. Initial DNA cleavage–religation reactions were allowed to come to equilibrium and were then diluted 20-fold with reaction buffer. Levels of DNA cleavage at time 0 were set to 100%. Error bars represent the SD of at least three independent experiments.

with the WT and resistant enzymes. Finally, in all cases, 8-methyl-moxi induced the most stable cleavage complexes. Again, this finding correlates with the high activity of 8-methyl-moxi.

Summary

As fluoroquinolones become more important for the treatment of tuberculosis and resistance becomes more prevalent, there is a critical need to understand the binding interactions and mechanistic details of quinolone-based agents with *M. tuberculosis* gyrase to develop new agents with high activity against WT and resistant disease. Thus, we analyzed interactions between quinolone-based agents and WT *M. tuberculosis* gyrase and resistant mutants. The interaction of current fluoroquinolones with gyrase is governed predominantly by a critical water–metal ion bridge. However, in contrast to most bacterial type II enzymes, *M. tuberculosis* gyrase uses only one amino acid to anchor the bridge. The most common resistance-conferring mutations in gyrase disrupt the bridge interaction between fluoroquinolones and the enzyme. When bridge function is compromised, resistance can be overcome, at least in part, by the introduction of specific groups at the C7 and C8 positions. By dissecting fluoroquinolone–enzyme interactions, we determined that an 8-methyl derivative of moxifloxacin induces high levels of stable cleavage complexes with WT and common fluoroquinolone-resistant mutant enzymes. This chemical biology approach to understanding the basis for fluoroquinolone–enzyme interactions has the potential to guide the future development of novel agents with improved activity against tuberculosis.

Methods

Materials and Enzymes. Ciprofloxacin and moxifloxacin were obtained from LKT Laboratories. 8-Methyl-cipro, 8-methoxy-cipro, 8-H-moxi, 8-methyl-moxi, 8-H-3'-(AM)P-FQ, 8-H-3'-(AM)P-dione, 8-methyl-3'-(AM)P-FQ, and 8-methyl-3'-(AM)P-dione were synthesized as reported previously (32). Ciprofloxacin-based compounds were stored at -20°C as 40 mM stock solutions in 0.1 N NaOH and diluted fivefold with 10 mM Tris-HCl (pH 7.9) immediately before use. All other compounds were stored at 4°C as 20 mM stock solutions in

100% DMSO. Table S1 contains the full chemical, library, and abbreviated names for the compounds used in this study. Other chemicals were of analytical reagent grade.

The coding regions for residues 2–675 of GyrB and 2–838 of GyrA from *M. tuberculosis* were amplified from genomic DNA (American Type Culture Collection strain H37Rv) and individually cloned into a pET-28b derivative containing an N-terminal, tobacco etch virus (TEV) protease-cleavable hexahistidine tag. Mutant GyrA vectors were generated by QuikChange (Agilent). Proteins were overexpressed in *E. coli* strain Rosetta 2 pLys5 (EMD Millipore) by growing cells at 30 °C until log phase, then inducing with 1 mM isopropyl- β -D-thiogalactopyranoside for 3 h. Cells were harvested by centrifugation and resuspended in buffer A [20 mM Tris-HCl (pH 7.9), 500 mM NaCl, 5 mM imidazole, and 10% (vol/vol) glycerol with protease inhibitors] and then frozen dropwise in liquid nitrogen.

Cells were sonicated and centrifuged, and the clarified lysate was passed over a HisTrap HP column (GE Healthcare). His-tagged protein was eluted with buffer B [20 mM Tris-HCl (pH 7.9), 500 mM NaCl, 250 mM imidazole, and 10% glycerol with protease inhibitors] and then concentrated and exchanged into buffer A overnight at 4 °C in the presence of His-tagged TEV protease. This mixture was passed over a HisTrap HP column, and the flow-through was collected, concentrated, and run over an S-300 gel filtration column (GE Healthcare) in buffer C [50 mM Tris-HCl (pH 7.9), 500 mM KCl, 2 mM β -mercaptoethanol, and 10% (vol/vol) glycerol]. Peak fractions were pooled, concentrated, and diluted into a high-glycerol variant of buffer C so that the final glycerol concentration reached 30% (vol/vol) before storage at –80 °C.

Human topoisomerase II α was expressed in yeast and purified as described by Kingma et al. (48).

Negatively supercoiled pBR322 plasmid DNA was prepared from *E. coli* by using a Plasmid Mega Kit (Qiagen) as described by the manufacturer. Relaxed pBR322 plasmid DNA was generated by treatment with topoisomerase I for 30 min as previously described (49), followed by phenol-chloroform-isoamyl alcohol extraction, ethanol precipitation, and resuspension in 5 mM Tris-HCl (pH 8.5) and 500 μ M EDTA.

DNA Supercoiling. DNA supercoiling reactions (20 μ L) contained 25 nM WT or mutant gyrase and 5 nM relaxed pBR322 in reaction buffer [10 mM Tris-HCl (pH 7.5), 40 mM KCl, 0.1 mg/mL BSA, 6 mM MgCl₂, 10% (vol/vol) glycerol, and 0.5 mM DTT] supplemented with 1 mM ATP and were incubated at 37 °C. DNA supercoiling was stopped at times from 0 to 45 min by the addition of 3 μ L of 0.77% SDS and 77.5 mM EDTA. Samples were mixed with 2 μ L of agarose gel loading buffer [60% (wt/vol) sucrose, 10 mM Tris-HCl (pH 7.9), 0.5% bromophenol blue, and 0.5% xylene cyanol FF], heated at 45 °C for 5 min, and subjected to electrophoresis in 1% agarose gels in 100 mM Tris-borate (pH 8.3) and 2 mM EDTA. Gels were stained with 0.75 μ g/mL ethidium bromide for 30 min. DNA bands were visualized with medium-range UV light and quantified by using an Alpha Innotech digital imaging system.

DNA Cleavage. DNA cleavage reactions were carried out using the procedure of Fortune and Osheroff as adapted by Aldred et al. (20, 50). Reactions with the bacterial enzyme contained 100 nM WT or mutant gyrase and 10 nM

negatively supercoiled pBR322 in a total of 20 μ L of reaction buffer. Reactions with the human enzyme contained 110 nM human topoisomerase II α and 10 nM negatively supercoiled pBR322 in a total of 20 μ L of 10 mM Tris-HCl (pH 7.9), 5 mM MgCl₂, 100 mM KCl, 100 μ M EDTA, 25 μ M DTT, and 2.5% (vol/vol) glycerol. All reaction mixtures were incubated at 37 °C for 10 min, and enzyme–DNA cleavage complexes were trapped by the addition of 2 μ L of 5% (wt/vol) SDS followed by 2 μ L of 250 mM EDTA (pH 8.0). Proteinase K (2 μ L of a 0.8 mg/mL solution) was added, and samples were incubated at 45 °C for 45 min to digest the enzyme. Samples were mixed with 2 μ L of agarose gel loading buffer, heated at 45 °C for 5 min, and subjected to electrophoresis in 1% agarose gels in 40 mM Tris-acetate (pH 8.3) and 2 mM EDTA containing 0.5 μ g/mL ethidium bromide. DNA bands were visualized as described earlier. DNA cleavage was monitored by the conversion of supercoiled plasmid to linear molecules.

Assays that monitored gyrase-mediated DNA cleavage in the absence of drugs substituted 6 mM CaCl₂ for 6 mM MgCl₂ in the reaction buffer. Assays that assessed DNA cleavage in the presence of drugs contained 0–100 μ M compound. In some reactions, the concentration dependence of MgCl₂ was examined or the divalent metal ion was replaced with 2.5 mM MnCl₂.

For assays that monitored competition between two drugs, the compounds were added simultaneously to reaction mixtures, and the final concentrations of the compounds are indicated. In these competition assays, the level of cleavage seen with the corresponding concentration of the competing drug (in the absence of the drug held at a constant concentration) was used as a baseline and was subtracted from the cleavage level seen in the presence of both compounds.

Persistence of Gyrase–DNA Cleavage Complexes. The persistence of gyrase–DNA cleavage complexes established in the presence of drugs was determined by using the procedure of Gentry et al. as adapted by Aldred et al. (20, 51). Initial reaction mixtures contained 500 nM WT or mutant gyrase, 50 nM DNA, and 100 μ M ciprofloxacin, 50 μ M 8-methoxy-cipro, 50 μ M moxifloxacin, or 10 μ M 8-methyl-moxi in a total of 20 μ L of reaction buffer. Quinolone concentrations that yielded similar levels of DNA cleavage were used. Reaction mixtures were incubated at 37 °C for 10 min and then diluted 20-fold with reaction buffer warmed to 37 °C. Samples (20 μ L) were removed at times ranging from 0 to 60 min for the mutant enzymes or 0–180 min for the WT enzyme, and DNA cleavage was stopped with 2 μ L of 5% (wt/vol) SDS followed by 2 μ L of 250 mM EDTA (pH 8.0). Samples were digested with proteinase K and processed as described earlier for DNA cleavage assays. Levels of DNA cleavage were set to 100% at time 0, and the persistence of cleavage complexes was determined by the loss of the linear reaction product over time.

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