Quinolones are the most active oral antibacterials in clinical use and act by increasing DNA cleavage mediated by prokaryotic type II topoisomerase. Although topoisomerase IV appears to be the primary cytotoxic target for most quinolones in Gram-positive bacteria, interactions between the enzyme and these drugs are poorly understood. Therefore, the effects of ciprofloxacin on the DNA cleavage and religation reactions of *Staphylococcus aureus* topoisomerase IV were characterized. Ciprofloxacin doubled DNA scission at 150 nM drug and increased cleavage 9-fold at 5 μM. Furthermore, it dramatically inhibited rates of DNA religation mediated by *S. aureus* topoisomerase IV. This inhibition of religation is in marked contrast to the effects of antineoplastic quinolones on eukaryotic topoisomerase II, and suggests that the mechanistic basis for quinolone action against type II topoisomerases has not been maintained across evolutionary boundaries. The apparent change in quinolone mechanism was not caused by an overt difference in the drug interaction domain on topoisomerase IV. Therefore, we propose that the mechanistic basis for quinolone action is regulated by subtle changes in drug orientation within the enzyme-drug-DNA ternary complex rather than gross differences in the site of drug binding.

Quinolone antibacterials were first synthesized over 30 years ago (1–4). Although the founding member of this drug class, nalidixic acid, had limited medical applications, its discovery spawned tremendous interest in the clinical potential of these compounds. Since that initial breakthrough, the use of quinolone-based drugs to treat bacterial infections in humans has grown considerably (2–6). In fact, this class is now the most active and broad spectrum family of oral antibacterial agents in clinical use (2–5).

Quinolones are targeted to the prokaryotic type II topoisomerasers, DNA gyrase and topoisomerase IV (6–20). These enzymes are essential to all bacterial species and play fundamental roles in most DNA processes (20–22). DNA gyrase, the only known topoisomerase that can actively underwind nucleic acids (23), is required for the maintenance of superhelical density in the bacterial chromosome and relieves torsional stress that accumulates in front of replication forks (20, 22, 24–27). Topoisomerase IV is responsible for unlinking newly replicated daughter chromosomes and resolving knots that result from recombination events (9, 20, 28, 29). Although these enzymes play different roles in the cell, they both alter the topological state of nucleic acids by passing a double helix through a transient break that they generate in a separate DNA segment (19, 20, 22, 26, 27).

Quinolones do not kill bacterial cells by blocking the essential functions of type II topoisomerases. Rather, they increase the cellular concentration of covalent topoisomerase-cleaved DNA complexes that are intermediates in the DNA strand passage reactions of these enzymes (4, 5, 10, 19, 20, 24, 26, 30–32). This action generates high levels of double-stranded breaks in the chromosomes of treated bacteria, triggers the SOS response, and ultimately induces cell death (4, 20, 26, 30, 32).

Before topoisomerase IV was discovered in 1990, DNA gyrase was believed to be the only significant target for quinolones in bacterial cells. To a great extent, this has proven true for Gram-negative species (4–8, 20). Although topoisomerase IV is a secondary target for these compounds in *Escherichia coli*, the cellular consequences of its interaction with quinolones are revealed only in the presence of drug-resistant DNA gyrase mutants (4, 6, 12, 20). However, in Gram-positive species, topoisomerase IV appears to be the primary cytotoxic target for most quinolones (4–6, 11, 14–17, 20, 33). This change in target reflects the increased sensitivity of Gram-positive topoisomerase IV to quinolone-based compounds coupled with the naturally drug-resistant form of gyrase found in these bacteria (4, 20, 34).

Quinolones targeted to topoisomerase IV, for the first time, have opened many Gram-positive species to this family of potent antibacterial drugs. This has allowed improved treatment of many infections (especially those of the respiratory tract) that were resistant to other antibiotics (4–6, 35). Although Gram-positive topoisomerase IV has become an important new target for antibacterial drug discovery, its interactions with quinolones are poorly understood.

Therefore, the present study characterized the effects of quinolones on the DNA cleavage/religation activity of topoisomerase IV from *Staphylococcus aureus*. In marked contrast to the actions of antineoplastic quinolones against eukaryotic type II topoisomerases (which increase levels of DNA breakage by stimulating the forward scission reaction), quinolones act primarily by inhibiting the ability of topoisomerase IV to relegate cleaved DNA molecules. Even though the functional drug
interaction domain on type II topoisomerases has been maintained from bacterial to eukaryotic species, it appears that the mechanistic basis for quinolone action has not been conserved across evolutionary boundaries.

**EXPERIMENTAL PROCEDURES**

Topoisomerase IV was cloned from *S. aureus* 4220, overexpressed as the separate subunits (GrLA and GrLB) in *E. coli*, and purified by a modification of the protocol of Hallett *et al.* (36). Briefly, cells from log-phase cultures were pelleted, resuspended in TED buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 5 mM dithiothreitol), lysed by sonication, and centrifuged at 18,000 × g for 30 min. Topoisomerase IV subunits were purified from both the soluble fraction and the cell pellet. The two extracts were combined and dialyzed against TEDG buffer (TED plus 10% glycerol) and the subunits were purified by the method of Hallett *et al.* (36) with the following changes. In the first step, the crude fraction was applied to a heparin-Sepharose column, washed with TEDG plus 0.25 M NaCl, followed by a linear gradient of 0.25 to 1.0 M NaCl in TEDG. Active fractions were combined, concentrated, brought to a final concentration of 1 M (NH₄)₂SO₄ and loaded on a phenyl-Superose FPLC column. Samples were eluted with a linear gradient starting at 1 M (NH₄)₂SO₄. Individual subunits were assayed for catalytic activity using an excess of the complementing specific activity of *S. aureus* topoisomerase IV was 3.1 × 10⁵ decatenation units/mg of protein (decatenating 200 ng of catenated kinetoplast DNA in 30 min at 37 °C). Human topoisomerase IIα was expressed in *Saccharomyces cerevisiae* (37) and purified by the protocol of Kingma *et al.* (38).

Etoposide and ciprofloxacin were obtained from Sigma. The quinolone CP-115,953 was synthesized at Pfizer Central Research. Etoposide was stored as a 10 mM stock in dimethyl sulfoxide at 4 °C. Ciprofloxacin and CP-115,953 were stored as 40 and 30 mM stock solutions, respectively, in 0.1 N NaOH at −20 °C, then diluted one-fifth with 10 mM Tris-HCl, pH 7.9, immediately prior to use. Tris and ethidium bromide were obtained from Sigma; SDS and proteinase K were from Merck; restriction endonucleases, calf intestine alkaline phosphatase, and T4 polynucleotide kinase were from New England Biolabs; ATP and [γ-3²P]ATP (6000 Ci/mmol) were from Amersham Pharmacia Biotech. All other chemicals were analytical reagent grade.

**Preparation of DNA Substrates**—Negatively supercoiled pBR322 DNA was isolated from *E. coli* as described previously (39). A uniquely end-labeled 564-base pair DNA substrate (residues 376–939 in the separate subunits (GrlA and GrlB) in *E. coli*, and purified by the protocol of Hallett *et al.* (36). Briefly, 5 mM negatively supercoiled pBR322 DNA was incubated with 15 nM topoisomerase IV in 20 μl of cleavage buffer (35 mM Tris-HCl, pH 7.9, 10 mM MgCl₂, 5 mM dithiothreitol, and 50 μg/ml bovine serum albumin) at 37 °C. Unless stated otherwise, reactions were carried out for 30 min. In drug competition assays, ciprofloxacin and etoposide were both present in the reaction mixture prior to the addition of topoisomerase IV, so that the enzyme was exposed simultaneously to both compounds. DNA cleavage reactions were stopped by the addition of SDS (0.5% final concentration) followed by EDTA (15 mM final concentration). Samples were digested with proteinase K (80 μg/ml final concentration) for 30 min at 45 °C. Following the addition of 60% sucrose, 0.5% bromphenol blue, and 0.5% xylene cyanol FF in 10 mM Tris-HCl, pH 7.9, DNA products were resolved by electrophoresis in 40% acrylamide gels in 40 mM Tris acetate, pH 8.3, 2 mM EDTA, and 0.5 mM ethidium bromide. DNA bands were visualized by UV light, photographed through Kodak 23A and 12 filters with Polaroid type 665 film, and quantitated by scanning negatives with an E-C apparatus model EC910 densitometer in conjunction with Hoefer GS-370 Data System software. The intensity of bands in the negative was proportional to the amount of DNA present. Double-stranded DNA breaks were monitored by the conversion of negatively supercoiled plasmid to linear molecules.

Sites of DNA cleavage were determined in the absence or presence of drugs using the protocol of Burden *et al.* (40). Assay mixtures contained 1.4 mM labeled linear 564-mer DNA (25 ng) and 6 nM topoisomerase IV in cleavage buffer, and were incubated at 37 °C for 15 min. Alternatively, 40 mM MgCl₂ contained 60 nM human topoisomerase IIα in 10 mM Tris-HCl, pH 7.9, 100 mM KC1, 5 mM MgCl₂, 0.1 mM EDTA, and 2.5% glycerol, and were incubated at 37 °C for 15 min. In both cases, DNA cleavage complexes were trapped by the addition of SDS and samples were digested with proteinase K in the presence of EDTA, as above. DNA cleavage products were precipitated twice with ethanol, dried, and resuspended in 40% formamide, 8.4 mM EDTA, 0.02% bromphenol blue, and 0.02% xylene cyanol FF. Samples were subjected to electrophoresis in 8% sequencing gels (42), fixed in 10% methanol, 10% acetic acid, and dried. Reaction products were visualized using a Molecular Dynamics PhosphorImager.

**Results**

Ciprofloxacin is the most widely prescribed quinolone in clinical use and represents one of the most active oral antibacterial agents currently available (2–6). It is cytotoxic to a broad range of bacteria and has well documented activity against topoisomerase IV from Gram-negative species (2, 4–6, 9, 12, 31). Therefore, it was chosen as the model quinolone for the present study.

Ciprofloxacin Stimulates DNA Cleavage Mediated by *S. aureus* Topoisomerase IV—As previously observed for its Gram-negative counterpart (31), topoisomerase IV from *S. aureus* displayed a robust DNA scission activity with negatively supercoiled substrates. A time course for DNA cleavage in the absence of drugs is shown in Fig. 1. The DNA cleavage/religation equilibrium of the enzyme was established within 30 s of the start of the reaction, and at a 3:1 ratio of topoisomerase IV:plasmid molecule, ~5% of the initial DNA substrate was cleaved. This is in contrast to human topoisomerase IIα, which cleaves <1% of the DNA molecules at a 30:1 ratio of enzyme:plasmid (31).

The DNA cleavage/religation equilibrium of *S. aureus* topoisomerase IV was profoundly affected by addition of ciprofloxacin (Fig. 1, inset, and Fig. 2). DNA scission increased as much as 9-fold in the presence of 5 μM drug. The concentration of quinolone required to double levels of cleavage (i.e. the CC₅₀ value) was ~150 nM. The effect of ciprofloxacin on the Gram-positive enzyme was even greater than that previously observed for *E. coli* topoisomerase IV (31). With this latter en-
zyme, cleavage levels increased only 4.5-fold, and ~500 nM quinolone was required to double scission (Fig. 2).

Maximal levels of quinolone-induced DNA cleavage are attained within 1–2 min in the presence of eukaryotic type II topoisomerases from Drosophila and humans (not shown). By comparison, it takes DNA gyrase ~1 h to re-establish its cleavage/religation equilibrium when ciprofloxacin is included in reaction mixtures (43). S. aureus topoisomerase IV is intermediate to these two enzymes; maximal levels of DNA scission were observed within 10 min following incubation with ciprofloxacin (Fig. 3).

Quinolones Act by Inhibiting DNA Religation Mediated by S. aureus Topoisomerase IV—As determined by their effects on DNA religation, drugs enhance DNA cleavage mediated by type II topoisomerases by two alternative (but not mutually exclusive) mechanisms (44, 45). While some drug classes strongly inhibit enzyme-mediated DNA religation, others have little effect on this reaction. Agents in this latter category presumably act by stimulating the forward rate of DNA cleavage.

Specific members of the quinolone family (typified by CP-115,953, which is shown with ciprofloxacin in Fig. 4) display antineoplastic activity and are potent enhancers of DNA scission mediated by eukaryotic type II topoisomerases (31, 46–55). These drugs show little or no ability to inhibit DNA religation mediated by these enzymes (47, 48). Thus, it has been suggested that antineoplastic quinolones act primarily by stimulating the forward DNA scission reaction.

In contrast, antibacterial quinolones have a greater effect on the DNA religation reaction of Gram-negative topoisomerase IV (31). When included in reaction mixtures, levels of ciprofloxacin that stimulated DNA cleavage ~4.5-fold decreased rates of religation mediated by E. coli topoisomerase IV ~2-fold (31). This finding suggests that inhibition of DNA religation by quinolones plays a more prominent role in prokaryotic species.

Since ciprofloxacin has a greater effect on the DNA cleavage activity of S. aureus topoisomerase IV than it does with the Gram-negative enzyme, the ability of this quinolone to inhibit DNA religation mediated by the Gram-positive enzyme was examined. A temperature shift assay was used for comparative studies (31). This assay is based on the finding that type II topoisomerases can religate, but not cleave DNA at suboptimal temperatures (56, 57).

Ciprofloxacin dramatically reduced levels of DNA religation in this assay (Fig. 5). At a drug concentration (5 μM) that stimulated DNA cleavage ~9-fold, rates of religation decreased ~7-fold. This is in marked contrast to the effects of antineoplastic quinolones on eukaryotic topoisomerase II, which display little to no inhibition of religation in this assay (47, 48).

Therefore, it appears that ciprofloxacin enhances DNA scission mediated by S. aureus topoisomerase IV primarily by inhibiting the ability of the enzyme to religate cleaved DNA molecules.

A caveat to the above conclusion is the fact that reactions with prokaryotic and eukaryotic type II topoisomerases utilized different members of the quinolone family. While ciprofloxacin contains an aliphatic piperazine ring at the C-7 position, CP-115,953 has a planar aromatic hydroxyphenyl substituent at this position (Fig. 4). Therefore, it is possible that findings with the bacterial type II topoisomerases reflect differences in the drug congeners employed, rather than evolutionary changes in quinolone mechanism.

To address this critical issue, the effects of CP-115,953 on the DNA cleavage/religation reaction of S. aureus topoisomerase IV were examined (Fig. 6). Results were strikingly similar to those observed for ciprofloxacin. CP-115,953 enhanced DNA scission >7-fold with a CC50 of ~160 nM (left). Moreover, when included in religation assays, CP-115,953 decreased reaction rates ~7-fold (right). These findings demonstrate that the pronounced quinolone inhibition of DNA religation mediated by Gram-positive topoisomerase IV reflects an inherent property of the drug-enzyme interaction rather than a difference in drug congeners.
gyrase that confers resistance to quinolones in clinical isolates of Gram-negative bacteria (i.e. Ser → Trp at position 83 in GyrA) (5, 18, 19) also confers resistance to antineoplastic quinolones when introduced at the corresponding residue in yeast topoisomerase II (58). Second, the antibacterial quinolone ciprofloxacin displays a weak ability to stimulate DNA scission mediated by eukaryotic topoisomerase II and acts as a competitive inhibitor of cleavage-enhancing anticancer drugs targeted to the enzyme (46, 53, 59).

Genetic evidence suggests that the site of quinoline binding on topoisomerase IV is comparable to that of DNA gyrase. Indeed, a similar spectrum of point mutations confers drug resistance to both enzymes (4, 6, 18–20). However, given the striking difference in the mechanistic basis for quinolone action on topoisomerase IV is comparable to that of DNA gyrase. Indeed, a similar spectrum of point mutations confers drug resistance to both enzymes (4, 6, 18–20). However, given the striking difference in the mechanistic basis for quinolone action on topoisomerase IV.

The drug increased levels of DNA scission ~2.5-fold (Fig. 8A), and shared many sites of cleavage enhancement with the antibacterial quinolone ciprofloxacin or the antineoplastic quinolone CP-115,953 (Fig. 7). Similar DNA cleavage patterns were also observed for CP-115,953 and etoposide in reactions that contained human topoisomerase IIα (Fig. 7, right).

Second, drug competition experiments were carried out to further define relationships between the quinolone and etoposide interaction domains on S. aureus topoisomerase IV. As seen in Fig. 8B, saturating concentrations of etoposide diminished the ability of ciprofloxacin to stimulate DNA scission ~50%. To confirm this finding, drug competition was assessed at the site-specific level. Experiments took advantage of the small subset of cleavage sites that were specific for quinolones and were not induced by etoposide. Representative sites are labeled I, 2, and 3 in Fig. 7. In the presence of etoposide, ciprofloxacin-induced scission at these sites dropped in a concentration-dependent manner (Fig. 8C). These results suggest that etoposide and ciprofloxacin share a common binding site on topoisomerase IV.

Third, the sensitivity of two common quinoline resistance mutants of S. aureus topoisomerase IV (Ser^{80} → Phe and Glu^{84} → Lys in the GrlA subunit) to etoposide was examined (5, 69). As seen in Table I, both mutations displayed cross-resistance to the anticancer drug.

Taken together, the above findings strongly suggest that the drug interaction domain on type II topoisomerases has been conserved throughout the evolution from prokaryotic to eukaryotic species. Therefore, it appears likely that the inhibition of topoisomerase IV-mediated DNA religation by quinolones reflects a subtle change in the enzyme-drug-DNA ternary com-
Quinolones Inhibit Topoisomerase IV-mediated DNA Religation

35931

Quinolones represent an important class of antibacterial agents that act by increasing levels of DNA cleavage mediated by prokaryotic type II topoisomerases (4, 5, 10, 19, 24, 26, 30, 31). In Gram-negative bacteria, quinolones are targeted primarily to DNA gyrase; topoisomerase IV serves only as a secondary target (4–9, 12, 20). However, in Gram-positive species, topoisomerase IV appears to be the primary cytotoxic target for most members of this drug family (4–6, 11, 14–16, 20, 33). Despite the importance of Gram-positive topoisomerase IV as a significant new drug target, its interactions with quinolones are not well understood. Therefore, the present study characterized the effects of quinolones on the DNA cleavage and religation reactions mediated by S. aureus topoisomerase IV.

Ciprofloxacin displayed high activity against the Gram-positive enzyme. As determined by DNA cleavage assays, the drug was more potent (>3-fold) and more efficacious (~2-fold) than it was against E. coli topoisomerase IV (31). Furthermore, quinolones inhibited DNA religation mediated by S. aureus topoisomerase IV to a greater extent than observed for any other type II enzyme (31, 45, 48, 51, 57). In fact, the level of inhibition was large enough to account for essentially all of the DNA cleavage enhancement induced by these drugs. This finding stands apart from those reported for the actions of antineoplastic quinolones against eukaryotic topoisomerase II (in which little inhibition was observed) (45, 48, 51, 57) or E. coli topoisomerase IV (in which ~50% of the DNA cleavage enhancement could be attributed to decreases in religation rates) (31). These results suggest that the mechanistic basis for quinolone action against type II topoisomerases is not conserved across evolutionary boundaries. Rather, as drug potency increases across species, it appears that inhibition of DNA religation plays a considerably more prominent role.

The apparent change in quinolone mechanism is not caused by an overt difference in the drug interaction domain on S. aureus topoisomerase IV. All available data suggests that the physical location of drug binding has been maintained throughout the evolution of the type II enzyme. Therefore, the following model is proposed to explain the inhibition of topoisomerase IV-mediated DNA religation by quinolones.

The model for quinolone action is based on the premise that stimulation of the forward DNA cleavage reaction and inhibition of the reverse DNA religation reaction by topoisomerase-targeted drugs do not actually represent two separate mechanisms; rather they reflect the two extremes of a mechanistic continuum. This model is based on the “positional poison model” (64, 65) and centers on two major points. First, it is postulated that quinolones increase enzyme-mediated DNA scission by distorting the double helix proximal to the points of cleavage (64, 65). This aspect of the hypothesis is supported by studies that localized drug-DNA cross-linking within the topoisomerase-drug-DNA ternary complex (66) and the positional specificity of cleavage-enhancing drugs and DNA lesions (60, 61, 64). In addition, chemical oxidation studies by Marians and Hiasa (67) have shown that the quinolone norfloxacin deforms DNA specifically within a cleavage site utilized by E. coli topoisomerase IV (67). Moreover, this distortion abuts the nucleotide bond cut by the enzyme.

Second, the location of quinolone-induced DNA distortion within the ternary complex determines whether the drug acts primarily by enhancing rates of DNA cleavage or by inhibiting DNA religation. When type II topoisomerases cleave DNA, they become covalently joined to the newly generated 5'-phosphate (22, 45, 56, 68). Because of this enzyme-DNA linkage, base pairing within the 4-base cleavage overhang is not required to promote efficient DNA religation (69). Therefore, when drug-induced distortion is encompassed completely within the cleavage overhang, rates of religation are unaffected (69). In contrast to the 5' terminus, the 3'-hydroxyl generated by cleavage is not covalently attached to the enzyme (22, 70, 71). Therefore, when DNA distortion induced by quinolones alters the orientation of the 3'-hydroxyl such that it can no longer be correctly positioned by the enzyme, the DNA religation reaction is inhibited. A similar inhibition could also be envisioned if the DNA-bound quinolone physically occluded the 3'-hydroxyl or blocked appropriate enzyme-nucleotide interactions by steric hindrance.

Using the above model as a guide, we suggest that the dramatically different effects of quinolones on DNA religation mediated by eukaryotic and prokaryotic type II topoisomerases actually result from a subtle change in the alignment of quinolones within the ternary complex. In eukaryotic systems, quin-
Quinolones Inhibit Topoisomerase IV-mediated DNA Religation

clone-induced distortion is located entirely within the cleavage overhang; thus these drugs do not affect DNA religation mediated by topoisomerase II. However, in bacterial systems, the position of DNA-bound quinolone is shifted so that it also affects the orientation of the 3'-hydroxyl (or interactions of the enzyme with the 3'-hydroxyl) and inhibits DNA religation. Since the most severe inhibition was observed with S. aureus topoisomerase IV, it is further suggested that the effects of quinolones on the position of the 3'-hydroxyl are more significant for the Gram-positive enzyme than they are for E. coli topoisomerase IV.

Among all the drug classes that target type II topoisomerases, quinolones are the only family that includes members display high activity against enzymes from prokaryotic and eukaryotic sources (4–6, 45, 51, 52). For example, the quinolone CP-115,953 is a potent enhancer of DNA cleavage mediated by such varied enzymes as E. coli DNA gyrase (47), Gram-positive and -negative topoisomerase IV (31), and topoisomerase II from thermophilic archaeabacteria (72), slime mold (73), yeast (74), Drosophila (47), and mammala (53, 75). This broad range of affected species affords a unique opportunity to examine drug mechanism and enzyme interactions over widely divergent evolutionary boundaries. Results of the present study strongly suggest that the mechanistic basis for quinolone action has not been conserved over the course of evolution. This finding underscores the universal application of drug mechanism from species to species and argues for further mechanistic studies with divergent type II topoisomerases.

Acknowledgments—We are grateful to Dr. Paul R. McGuirk (Pfizer Central Research) for the synthesis of CP-115,953 and Dr. D. Andrew Burden, Susan D. Cline, and John M. Fortune for critical reading of the manuscript.

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