Quinolones: Action and Resistance Updated

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Abstract: The quinolones trap DNA gyrase and DNA topoisomerase IV on DNA as complexes in which the DNA is broken but constrained by protein. Early studies suggested that drug binding occurs largely along helix-4 of the GyrA (gyrase) and ParC (topoisomerase IV) proteins. However, recent X-ray crystallography shows drug intercalating between the -1 and +1 nucleotides of cut DNA, with only one end of the drug extending to helix-4. These two models may reflect distinct structural steps in complex formation. A consequence of drug-enzyme-DNA complex formation is reversible inhibition of DNA replication; cell death arises from subsequent events in which bacterial chromosomes are fragmented through two poorly understood pathways. In one pathway, chromosome fragmentation stimulates excessive accumulation of highly toxic reactive oxygen species that are responsible for cell death. Quinolone resistance arises stepwise through selective amplification of mutants when drug concentrations are above the MIC and below the MPC, as observed with static agar plate assays, dynamic *in vitro* systems, and experimental infection of rabbits. The gap between MIC and MPC can be narrowed by compound design that should restrict the emergence of resistance. Resistance is likely to become increasingly important, since three types of plasmid-borne resistance have been reported.

INTRODUCTION

The quinolones are broad-spectrum antibacterial agents that are receiving increasing attention as resistance develops to other compounds. Unfortunately, the quinolones are also losing their utility due to bacterial resistance, which creates a sense of urgency to develop new, more effective derivatives. As a result, biochemical insights continue to emerge, and we can now begin to discuss crystal structures of drug-target-DNA complexes. Our understanding of intracellular quinolone action is also deepening. For example, evidence is accumulating that lethal action is due to chromosome fragmentation and the resulting surge in reactive oxygen species (ROS). While finding new quinolone derivatives has continued along conventional lines that seek low MIC, that effort is expanding to include identification of compounds having good activity with mutants resistant to existing compounds. We expect studies with fluoroquinolone resistance to eventually lead regulatory agencies to add anti-mutant properties to the evaluation of new compounds. These and other developments make an update of quinolone action and resistance timely.

We use the term quinolone in a generic sense that refers loosely to a class of inhibitors that includes naphthyridones, quinolones, quinazolines, isothiazoloquinolones, and related agents. These compounds have as their targets two essential bacterial enzymes, DNA gyrase (topoisomerase II) [1] and DNA topoisomerase IV [2]. The two enzymes, each of which contains 4 subunits (2 GyrA or ParC and 2 GyrB or ParE), act by passing one region of duplex DNA through another [3-6]; during that process, the quinolones form complexes with enzyme and DNA [1, 7]. The DNA moiety in the complex is broken, as revealed by detection of fragmented DNA following addition of protease, ionic detergent (sodium dodecyl sulfate, SDS), or both to quinolone-containing reaction mixtures or lysates from quinolone-treated bacterial cells [1, 7, 8]. The complexes are called "cleaved" or "cleavable" to indicate the presence of broken DNA that is covalently attached to the enzyme at the 5' ends. Chromosomal DNA remains supercoiled when obtained from cells treated with quinolones at bacteriostatic concentrations, provided that the complexes are kept intact by omission of protein denaturants from cell lysis procedures [8]. The presence of supercoils indicates that the DNA breaks in the complexes are constrained in a way that prevents the rotation of DNA ends that would otherwise relax supercoils. However, when cells are treated with lethal drug concentrations, the supercoils are absent, indicating release of the DNA ends from the complexes. That release is expected to fragment chromosomes.

The hallmark of quinolone action is formation of cleaved complexes. *In vitro*, the complexes block movement of replication forks and transcription complexes, thereby inhibiting bacterial growth [9-11]. Lethal action arises at higher quinolone concentrations in parallel with chromosome fragmentation. Thus, bacteriostatic action and rapid lethal effects are distinct. By normalizing lethal action to MIC, it is possible to minimize the contribution of factors, such as drug uptake and efflux, that would otherwise confound comparison of quinolones during studies of drug mechanism.

Cleaved complexes are also important for quinolone resistance, because the common resistance mutations inter-

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fere with drug binding [12]. However, quinolone resistance also arises from mutations that alter drug uptake, efflux, and structure [13-17]. Many of these mutations do not by themselves provide clinical resistance, but they may facilitate the stepwise accumulation of additional mutations [13, 18, 19]. Stepwise resistance distinguishes the emergence of quinolone resistance from the all-or-none phenomenon seen for rifampicin with *Escherichia coli* and *Staphylococcus aureus* [20]. It also underlies use of the mutant selection window hypothesis as a framework for suppressing the emergence of resistance (the hypothesis maintains that resistant mutant subpopulations are selectively enriched and amplified when drug concentrations fall in a range above the MIC for the susceptible population and below the MIC of the least susceptible mutant subpopulation, a value called the MPC). The selection window can be used to formulate dosing regimens, to choose compounds for therapy, and to design new agents.

Below we turn first to biochemical studies of cleaved complex formation. Knowledge gained from crystal structures is moving us toward an atomic description of the complexes, with current data appearing to require a two-step model. An underlying assumption of structural studies is that the type II topoisomerases have very similar structures; consequently, conclusions drawn with one enzyme are often applied to others. While this assumption is generally sound, the enzymes differ; in the second section we discuss the C-terminal domains of the GyrA and ParC proteins, regions where major differences between gyrase and topoisomerase IV appear. We then shift to biological consequences of cleaved complex formation: inhibition of DNA replication, chromo-some fragmentation, and accumulation of ROS. Recent studies of resistance include support for the mutant selection window hypothesis and the discovery of new quinolone-like compounds that exhibit excellent *in vitro* activity with mutants resistant to existing quinolones. We conclude with an update on the three types of plasmid-borne fluoroquinolone resistance. Readers interested in earlier reviews are referred to [21-27].

CRYSTAL STRUCTURES AND MODELS FOR CLEAVED COMPLEXES

For many years our understanding of quinolone action has been based on crystal structures of GyrA fragments [28] and eukaryotic topoisomerase II [29]. Such studies describe the portion of GyrA and ParC involved with the DNA breaks. Most of the attention focused on helix-4 because it is the location of amino acid substitutions generally associated with quinolone resistance and presumably drug binding. Since quinolones were not part of these structures, the work revealed little about the positioning of the drugs.

When the structure of a co-crystal of yeast topoisomerase II and DNA was solved [30], several striking features were seen. First, the topoisomerase forces a 150° bend in DNA upon binding to the G (gate)-segment of DNA [30]. Second, the central four base pairs of the binding site adopt an A-form conformation, whereas DNA at the outermost edges of the G-segment binding site is B-form. Third, large conformational changes of the enzyme take place upon its binding to DNA [30], a conclusion that supports earlier biochemical

work [31]. The conformational change creates a catalytic site having a DNA binding surface that extends across both protein protamers. This conformation positions the DNA backbone near a reactive tyrosine and a coordinated magnesium ion thought to be part of the DNA cleavage reaction.

Covariation between C-7-piperazinyl ring substituents and susceptibility to particular resistance substitutions suggested a drug-binding orientation (Fig. **1**). For example, with *Mycobacterium smegmatis*, a fluoroquinolone with a C-7-piperazinyl-N-linked ethyl moiety was less active against a Gly-81 to Cys variant (we use the *E. coli* numbering system for simplicity) than a similar quinolone with a C-linked ethyl [32]; amino acid substitutions at other positions in helix-4 failed to distinguish between the compounds. Since position 81 is located at the N-terminus of helix-4, the idea arose that the C-7-distal end of the quinolone binds near the N-terminus of the helix. According to this hypothesis, the other (keto-carboxy) end of the quinolone would bind near amino acid positions 83 and 87, two positions where major resistance substitutions map. As a further test of this idea, we recently constructed a C-7 piperazinyl N-bromoacetyl derivative of ciprofloxacin (Cip-Br) that has intracellular properties consistent with crosslinking to Cys-81 (low MIC and irreversibility of inhibition of DNA synthesis that are specific to Cys-81 and the bromo compound; A.M. and M.M., unpublished observations). Our data are consistent with binding of quinolones to multiple points along helix-4 with the C-7 ring near position 81 (Fig. **1**).

A very different idea for drug binding recently arose from a crystal structure of a cleaved complex composed of the DNA-binding core of *Streptococcus pneumoniae* topoisomerase IV complexed with broken DNA and either clinafloxacin (Fig. **2A**) or moxifloxacin [33]. In this model, each fluoroquinolone molecule intercalates in the gap between the -1 and +1 nucleotide pairs of the cleaved DNA bound to the symmetrical topoisomerase IV heterodimer (Fig. **2B** shows binding of one clinafloxacin molecule). Interaction with the -1 nucleotide is consistent with the observation that an abasic site at the -1 position inhibits formation of quinolone-induced cleaved complex at the site (HH, unpublished observations). A characteristic feature of the DNA intercalation model is the interaction of the C-7 substituent of the quinolone with DNA base pairs rather than with amino acid 81, which is far from the DNA moiety.

In the DNA intercalation model (Fig. **2B**), the 3-carboxyl group of the fluoroquinolone rests on a platform composed of the amino terminus of helix-4 such that the 3-carboxyl contacts Ser-79 (position 83 in *E. coli* GyrA) and is in close proximity to Ser-80 (Ala-84 of GyrA). The carboxyl group of Asp-78 (82 in GyrA) is not resolved in the structure, but it may be close enough to the 3-carboxyl group of the fluoroquinolone to allow formation of a Mg^{2+} bridge, which has been suggested to be important for drug binding due to the Mg^{+2}-dependence of complex formation and reversal of DNA cleavage by EDTA [7]. Alternatively, the 3-carboxyl may participate in an electrostatic interaction with the guanidine group of Arg-118 (GyrA 121), which is also unresolved in the structure. Finally, one of the hydrogen atoms of the guanidine group of Arg-118 can form a hydro-
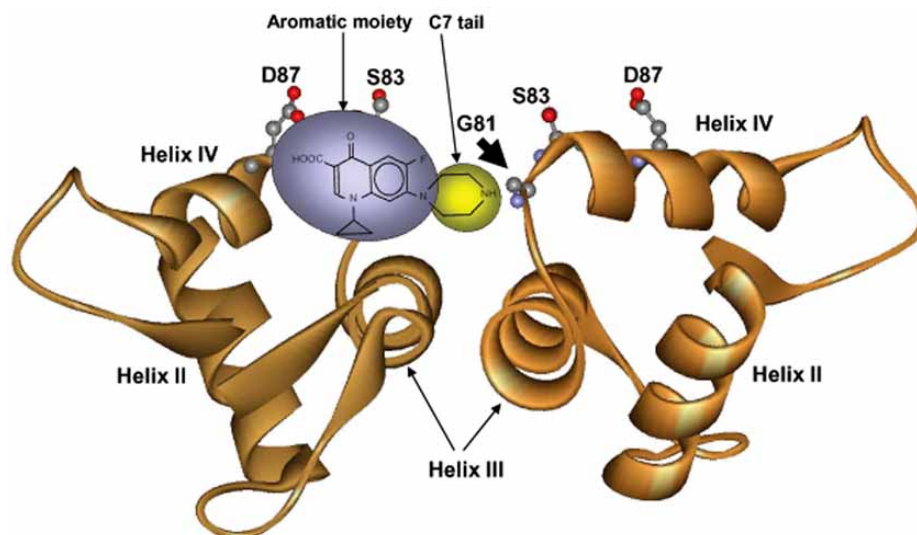
Fig. (1). Helix-4 quinolone-binding model. The DNA-gate region is shown for a GyrA-GyrA dimer to which one fluoroquinolone molecule is bound such that the distal end of the C-7 ring (C7 tail) is near GyrA position 81 of one GyrA subunit; carboxyl and keto oxygens are near GyrA positions 87 and 83, respectively, of the other subunit.

gen bond with the 4-keto group of the drug, which would strengthen binding.

The DNA intercalation model shows how drug binding could prevent the religation of DNA. It also explains the protective effect of some resistance mutations. For example, a substitution at Asp-78 (GyrA 82) would eliminate a putative Mg^{2+} bridge, thereby weakening drug binding; substitution at Gly-77 (GyrA 81) could introduce a bulky side chain that would sterically clash with the oxygen of the fluoroquinolone 3-carboxyl group and/or push away the side chain of Arg-118, thereby interfering with interaction of this residue with the fluoroquinolone. The close proximity of Arg-118 (GyrA 121) to the fluoroquinolone 3-carboxyl group suggests that an interaction there might be significant to drug binding, which should be reflected in the recovery of resistance substitutions. Such mutations are not common, perhaps due to the importance of the arginine residue for catalysis (this amino acid is highly conserved among type II topoisomerases). Substitutions of Ser-79 (GyrA 83), as well as those at position 80 (GyrA 84), are expected to reposition the drug molecule, thereby affecting other fluoroquinolone-protein interactions. The DNA intercalation model is also consistent with the protective effect of ParE substitutions at amino acids 435 (GyrB 426) and 456 (GyrB 447), which are located close to the bound drug.

The effects of several amino acid substitutions and drug structure variations are unexplained by the DNA inter-calation model. One example is the putative interaction of the C-7 substituent with position 81, as noted above. Another is substitution at position 83 (GyrA 87), which is highly protective from quinolone action. In the model, GyrA-87 substitutions are too far from the fluoroquinolone-binding site to interfere with drug action. The model also fails to explain effects of drug substituents at positions 1 and 8 that significantly alter both the antimicrobial activity and the drug-target binding constant [34]: in the X-ray structure, the moieties at positions 1 and 8 make no contact with either DNA or protein. In particular, the (-) isomer of ofloxacin

(levofloxacin) binds to bacterial gyrase about 10-12 times more efficiently than the (+) isomer [35], but modeling of ofloxacin onto the DNA intercalation structure provides no insight into the effects of the isomers. In addition, the role of the fluorine substitution at position 6, which significantly improves antibacterial activity, remains obscure. Laponogov *et al*. [33] suggest that the C-6 fluorine might influence charge distribution to favor stacking interactions with DNA bases. Finally, the protective effect of a substitution at Gly-77 (GyrA 81) for fluoroquinolones but not nalidixic acid [36] remains unexplained, as does the effect of the Cip-Br derivative when Cys is substituted for Gly at position 81. The latter is particularly problematic, since the DNA inter-calation model asserts that the C-7 piperazinyl ring, which we suggest can be crosslinked to GyrA Cys-81, stacks with DNA bases.

One way to accommodate the genetic-drug structure data described above with the intercalation model is to postulate that quinolone binding is a multi-step process involving structures in which drug binding is quite different. Indeed, quinolone binding is known to involve at least two steps, one that occurs before DNA cleavage and one that occurs after [37, 38]. Thus, Figs. **1** and **2** may describe two different steps in cleaved complex formation.

DIFFERENCES BETWEEN THE CARBOXYL-TERMINAL DOMAINS OF GYRA AND PARC

The two molecular targets of the quinolones, DNA gyrase and DNA topoisomerase IV, are homologous, and many interactions with quinolones are very similar for the two enzymes [25]. Indeed, all of the type II topoisomerases, except for topoisomerase VI of *Sulfolobus shibatae*, are highly conserved [21, 27]. However, each enzyme also exhibits a distinct catalytic preference that reflects its specialized intracellular function. For example, DNA gyrase is the only enzyme that can introduce negative supercoils into DNA, whereas topoisomerase IV relaxes negative
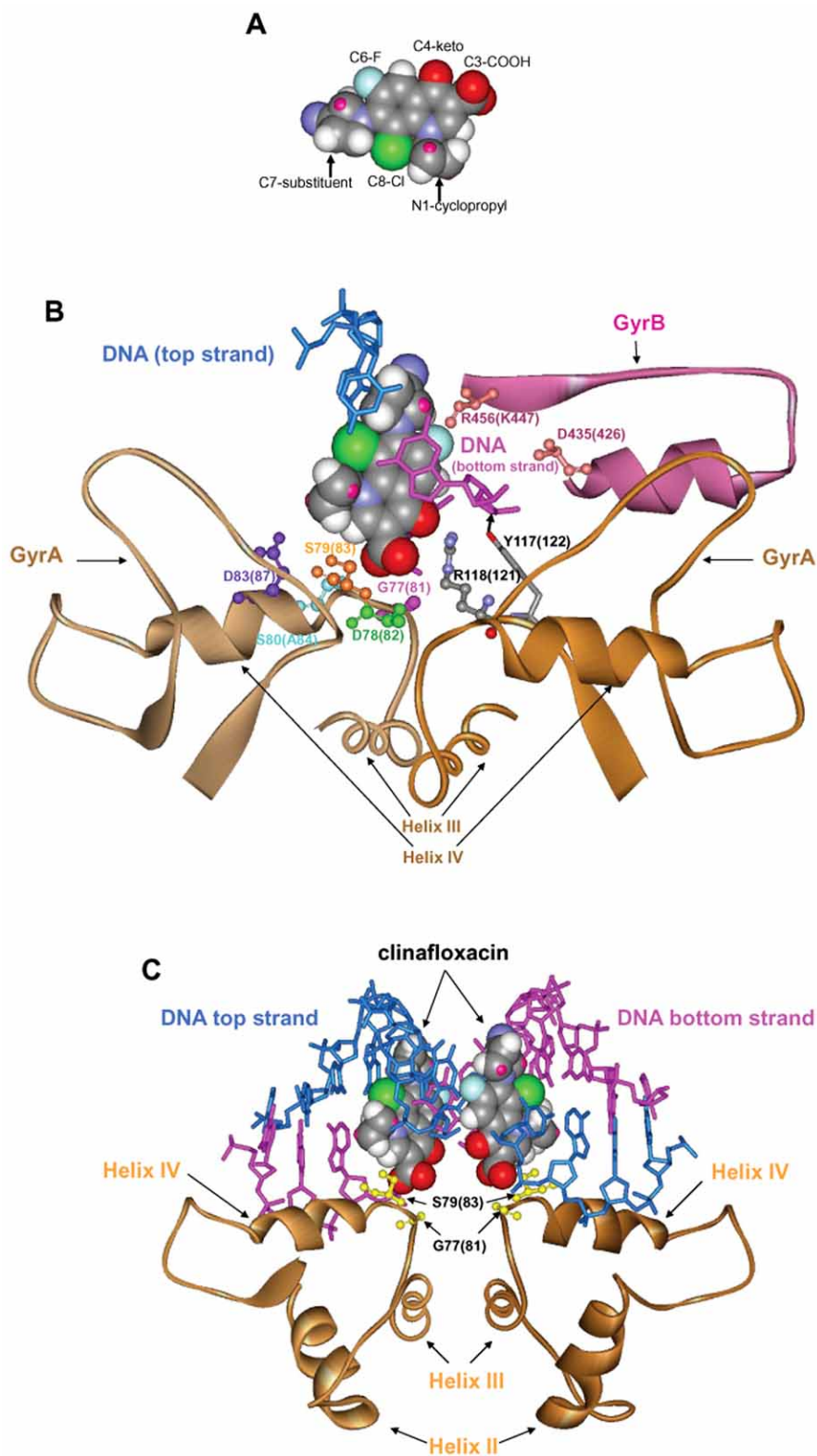
Fig. (2). DNA intercalation model for quinolone binding derived from clinafloxacin-topoisomerase IV-DNA crystallography. Panel **A**. Space-filling model for clinafloxacin. Panel **B**. Relative arrangement of one clinafloxacin molecule, cleaved DNA, and portions of topoisomerase IV in the co-crystal structure described by [33]. Panel **C**. Relative arrangement of two clinafloxacin molecules, cleaved DNA, and portions of topoisomerase IV in co-crystal structure described by [33]. Protein and DNA residues in immediate contact with, or in close proximity to FQ are indicated (ball & stick representation). ParC features (helixes III, IV) are shown in beige; a short region of ParE (maroon) shows location of ParE resistance substitutions. DNA residues flanking the drug molecules are shown in a stick representation (top strand, blue; bottom strand, magenta).

supercoils [39] and decatenates and unknots DNA [40-42]. The binding of topoisomerase IV to the G-segment DNA takes place only at the amino-terminal catalytic domain (NTD) of the ParC subunit, whereas GyrA binds to the G-segment at both its NTD and its carboxy-terminal domain (CTD). Binding at the CTD is thought to wrap the G-segment DNA around gyrase, thereby enabling the enzyme to catalyze DNA supercoiling [43, 44]. Thus, knowledge of CTD structure and function is important for understanding differences between gyrase and topoisomerase IV.

Recent structural studies show that a 35-kDa fragment of the CTD of *Borrelia burgdorferi* GyrA adopts a 6-bladed 'β-pinwheel' fold that is reminiscent of the 'β-propeller' fold [45]. Three other CTDs, the *Bacillus stearothermophilus* ParC CTD [46], the *Escherichia coli* GyrA CTD [47], and the *E. coli* ParC CTD [48], also adopt a β-pinwheel fold. While all GyrA CTDs possess 6 blades, ParC CTDs exhibit significant structural diversity [45, 48]: some ParC CTDs, such as that from *B. stearothermophilus*, adopt a 6-bladed β-pinwheel fold, whereas others, such as the *E. coli* ParC CTD, adopt a 5-bladed fold. Interestingly, the *B. stearothermophilus* ParC CTD superimposes well on the *E. coli* GyrA CTD [48, 47]. These structural studies of GyrA and ParC CTDs, together with phylogenetic data on gyrases and topoisomerase IVs, lead to a new picture of how bacterial type II topoisomerases are likely to have evolved [45, 48]. Gyrase had been considered to be a specialized enzyme that had evolved primarily to supercoil DNA [21, 27]. However, the specialized function of topoisomerase IV, incremental changes observed in the ParC proteins, and a wider distribution and greater conservation of GyrA than ParC, suggest that ParC CTDs are degenerate forms of the GyrA CTD and that topoisomerase IV evolved from gyrase [48]. The same conclusion is also reached by comparing GyrA and ParC NTDs [49].

Amino acid sequence alignment, supplemented with secondary structure predictions, reveals that the 7-amino-acid-long GyrA box, QRRGGKG [50], is the only motif unique to the GyrA CTD [45, 48]. Either deletion or alanine substitution in the GyrA box abolishes the ability of gyrase to wrap DNA around itself and to catalyze DNA supercoiling [51]. However, these mutations do not affect the relaxing and decatenating activities of gyrase. Thus, the GyrA box is essential for the unique (supercoiling) activity of gyrase.

The GyrA CTD, which is joined to the GyrA NTD through a flexible linker, moves between upper and lower positions during the catalytic cycle [52, 53]. The binding of the GyrA box to G-segment DNA may coordinate both the position of the GyrA CTD and the direction of G-segment DNA bending to allow gyrase to wrap DNA for the supercoiling reaction [51]. In contrast, the *E. coli* ParC protein has an ordered linker between its NTD and CTD, and the position of the ParC CTD remains fixed relative to the ParC NTD. That prevents the ParC CTD from binding to G-segment DNA [48]. Instead, the ParC CTD captures an incoming T (transfer)-segment DNA to catalyze either decatenation or relaxation.

As discussed below, the wrapping of DNA turns out to be important to the stability of cleaved complexes formed with

gyrase and the ability of the complexes to block replication fork progression [54, 55]. Thus, although the GyrA CTD does not directly interact with quinolone, the CTD appears to influence drug action. Topoisomerase IV, which does not wrap DNA, forms cleaved complexes that are intrinsically stable enough to cause replication fork arrest [54, 55].

BACTERIOSTATIC ACTION OF QUINOLONES

Replication Fork Arrest by Quinolone-Topoisomerase-DNA Complexes

Rapid inhibition of DNA replication is one of the more striking consequences of cleaved complex formation with quinolones [8, 56] and with inhibitors of eukaryotic DNA topoisomerases [57, 58]. In the case of camptothecin and eukaryotic topoisomerase I, collision of replication forks with cleaved complexes causes fork breakage and the release of lethal DNA breaks [59]. Since topoisomerases share general features, the possibility arose that a similar phenomenon would occur with quinolones and gyrase/topoisomerase IV. Indeed, irreversible collision of replication forks with quinolone-mediated complexes was thought to be the primary source of cell death [60]. However, other work with quinolones indicated that inhibition of replication is reversible [56]. Thus, the relationship between inhibition of DNA synthesis and cell death required additional investigation.

Several studies have been performed that clarify relationships between cell death and collision of replication forks with quinolone-mediated cleaved complexes. In one approach, cells were treated in ways that block cell death, and then the treatments were assessed for effects on quinolone-mediated inhibition of DNA synthesis or growth. For example, chloramphenicol and anaerobic growth prevent first-generation quinolones from killing *E. coli*. The former has little effect on quinolone-mediated inhibition of DNA synthesis [61], and the latter allows quinolones to form complexes that block growth, presumably by inhibiting replication and transcription [62, 63]. Thus, replication and cell death are distinct. Another approach was to block replication by means other than quinolone treatment and then determine whether the quinolones still kill cells. When such an experiment was performed with a temperature-sensitive *dnaB* mutant, stopping replication had little effect on the lethal activity of the quinolones [64]. A third approach was to reconstitute the collision between replication forks and quinolone-containing complexes *in vitro* and determine whether double-strand DNA breaks were generated or released [9]. They were not [9]. Consequently, the active DNA breakage and reunion activity of either gyrase or topoisomerase IV, which is required to arrest replication fork progression [9, 10, 54], does not cause the breakage of replication forks. Similar conclusions have been reached with topoisomerase II-targeting anticancer drugs using both *in vivo* and *in vitro* systems [65, 66]. We conclude that cleaved complexes composed of type II topoisomerases block replication fork movement without causing fork breakage and rapid cell death.

Since quinolone-induced cleaved complexes contain broken DNA, it was reasonable to assume that replication fork arrest would occur at any cleaved complex [22, 26, 67,

68, 69]. However, with *S. aureus* only a subset of quinolone-induced cleaved complexes appears to be physiologically competent for quinolone action [70], an observation that led us to examine how the stability of quinolone-induced cleaved complexes contributes to their ability to block replication fork movement.

One line of investigation showed that *S. aureus* gyrase requires high concentrations of potassium glutamate to wrap DNA, catalyze DNA supercoiling, and arrest replication fork progression *in vitro* [55]. Similar studies using an *E. coli* mutant gyrase that lacks the entire GyrA CTD (GyrA59), and thus cannot wrap DNA [43], showed that gyrase-mediated DNA wrapping is required for replication fork arrest [54]. Cleaved complexes formed with GyrA59 gyrase are also more sensitive to salt than those formed with the wild-type gyrase, and GyrA59 gyrase-quinolone-DNA ternary complexes readily disassociate from DNA. Thus, wrapping of DNA is required for the formation of gyrase-containing cleaved complexes that are stable enough to block replication fork progression.

Topoisomerase IV also forms cleaved complexes that arrest replication fork progression [54, 55], but topoisomerase IV does not wrap DNA. We found that quinolone-induced cleaved complexes formed with topoisomerase IV are more stable than those formed with GyrA59 gyrase [54]. Critical differences may exist between the GyrA and ParC NTDs with respect to their interactions with DNA and/or the quinolones in cleaved complexes. Another possibility is that the placement of the CTD near the tower domain of GyrA or ParC is important for the stability of cleaved complexes. Gyrase uses DNA wrapping to position the GyrA CTD near the tower domain [52, 53], whereas it is the ordered linker between the NTD and the CTD that places the ParC CTD near the tower [48].

Studies of helix-4 also bear on complex stability. As pointed out above, two mutational hotspots for quinolone resistance, Ser-83 and Asp-87, locate on GyrA helix-4 [28], and similar hotspots are found in analogous regions of ParC [22, 68]. Thus, helix-4 is probably a part of the quinolone-binding site, as pointed out in the crystal structure section above. Although DNA-binding domains, including helix-4, are highly conserved among gyrases and topoisomerase IVs, differences do occur. Swapping helix-4 of *E. coli* GyrA with that of *E. coli* ParC [71] and swapping an extended region around helix-4 of *E. coli* GyrA with that of *S. aureus* GyrA [72] reveal that subtle differences in amino acid residues in and/or around helix-4 affect the quinolone sensitivity of a topoisomerase. Furthermore, quinolone-induced cleaved complexes formed with a mutant topoisomerase IV containing helix-4 of *E. coli* GyrA are more sensitive to quinolone and more stable than those formed with topoisomerase IV; they are less sensitive and less stable than complexes formed with gyrase [71]. Thus, quinolone sensitivity of a topoisomerase correlates with the stability of the cleaved complex. Likewise, cytotoxicity of topoisomerase II-targeting anticancer drugs correlates with the stability of drug-induced cleaved complexes [73].

Double-Strand DNA Breaks Arising from Inhibition of Replication

While we have argued above that lethal replication fork breakage does not arise from collision of replication forks with cleaved complexes containing gyrase or topoisomerase IV, a small number of non-lethal (repairable) breaks probably arise. One line of evidence emerges from the signature response to quinolone treatment, the induction of the SOS regulon [74-77]. Quinolone-mediated induction of the SOS response requires the action of RecBCD, which in turn requires a free DNA end to load onto DNA. Consequently, it is likely that some double-strand breaks are generated by collision of replication forks with cleaved complexes. Indeed, in a plasmid model system where cleaved complex formation blocked replication, double-strand breaks were observed [78, 79].

It has been suggested that the double-strand DNA breaks arising after replication fork stalling are generated by a recombination nuclease [78, 79]. Interestingly, a significant portion of the double-strand breaks associated *in vivo* with cleaved complexes and blockage of replication fork progression are reversible [79]. When replication forks are stalled *in vivo*, they trigger 'replication restart' processes catalyzed by recombination proteins [80-83]. It is presently unclear which recombination proteins are involved in quinolone-induced generation of double-strand breaks. One candidate is the RuvABC complex. Since RuvAB can reverse a topoisomerase IV-quinolone-DNA ternary complex *in vitro* [84], it is possible that RuvAB may reverse and dissociate the cleaved complex at a stalled replication fork before RuvC cleaves DNA to generate a double-strand break [79]. While we consider it important to distinguish the few DNA breaks associated with replication fork arrest from the extensive chromosome fragmentation associated with cell death (discussed below), it is conceivable that some repair proteins are involved in both.

LETHAL ACTION OF QUINOLONES

Quinolone Generations and Pathways to Cell Death

The quinolones kill *E. coli* by two pathways. One is blocked by inhibitors of protein synthesis, such as chloramphenicol, and by anaerobic conditions. The second pathway is active even in the presence of chloramphenicol or the absence of oxygen. Four structural quinolone generations are distinguished by the effects of chloramphenicol and anaerobiosis on quinolone lethality [63]. First-generation compounds, such as nalidixic and oxolinic acids, are not lethal in the presence of chloramphenicol or during anaerobic growth; the second-generation agent norfloxacin fails to kill *E. coli* in the presence of chloramphenicol, but at high concentrations it kills cells growing anaerobically (norfloxacin also kills *E. coli* suspended in saline, while nalidixic acid does not). Ciprofloxacin, a third-generation compound, kills under both conditions but requires higher concentrations during anaerobiosis; the lethal activity of fourth generation C-8-methoxy derivatives, such as

PD161144, is affected little by chloramphenicol or anaerobic growth. These data fit with the idea that some compounds function more through one pathway than the other. The choice of pathway depends on quinolone concentration, since even the fourth-generation compounds are sensitive to chloramphenicol if quinolone concentrations are low enough.

The two lethal pathways are also observed in mycobacteria. For example, with *M. tuberculosis* gatifloxacin and moxifloxacin, two C-8-methoxy compounds, are indistinguishable when lethal activity is measured with growing cultures. However, when chloramphenicol is added, moxifloxacin is strikingly more active [85]. Moxifloxacin is also more active when growth of *M. bovis* BCG is arrested by treatment with nitric oxide [86]. Since the two fluoroquinolones differ only in their C-7 ring systems, we can begin to attribute death of non-growing cells to the C-7 substituent.

Chromosome Fragmentation

Since quinolone-enzyme-DNA complexes contain broken DNA and since chromosome fragmentation is likely to kill cells [87], we postulated that cell death arises from the release of DNA breaks from protein-mediated constraint existing in the cleaved complexes. The first evidence for this idea came from supercoiling studies with *E. coli* nucleoids, as pointed out in the introduction [61]. Treatment with lethal concentrations of oxolinic acid allowed relaxation of DNA supercoils, an event that failed to occur when cells were pretreated with chloramphenicol. In these experiments high concentrations of ethidium bromide, a DNA-intercalating agent, failed to introduce positive supercoils, indicating that the DNA relaxation arose from DNA breakage. Ciprofloxacin, a compound that kills cells in the presence of chloramphenicol, relaxed supercoils whether or not protein synthesis was blocked. This correlation between cell death and chromosome fragmentation was subsequently strengthened by sedimentation and viscometric measurements [88].

Insight into the chloramphenicol-insensitive lethal pathway came initially from work by Ikeda. His laboratory found a form of quinolone-stimulated illegitimate recombination that was attributed to gyrase subunit dissociation-reassociation [89, 90]. Quinolone-mediated gyrase subunit dissociation could explain lethality that is unaffected by chloramphenicol: in the cleaved complexes the quinolones might pry gyrase subunits apart and fragment chromosomes. This idea is supported by the ability of gatifloxacin to fragment isolated chromosomes in the presence of purified gyrase. Moreover, a GyrA A67S variant is killed by nalidixic acid in the presence of chloramphenicol [88], an event that does not occur with wild-type cells. In this variant an Ala residue expected to lie on the GyrA-GyrA dimer interface is substituted by Ser, a change that could weaken hydrophobic interactions and promote subunit dissociation. So far, no structural model of the cleaved complex explains lethal action.

The basis of chromosome fragmentation that requires ongoing protein synthesis is even less clearly defined. The three most obvious mechanisms for releasing DNA breaks from protein-mediated constraint are 1) protease digestion of gyrase, 2) nuclease-mediated cleavage on either side of the cleaved complex, and 3) protein denaturation. Once this fragmentation occurs, death arises from ROS, as described below.

Amplification of Lethal Action by Reactive Oxygen Species

Collins and co-workers recently discovered that hydroxyl radical concentrations are elevated in *E. coli* following treatment with several lethal antimicrobials, including norfloxacin [91, 92]. We subsequently found that when both *sodA* and *sodB* were deficient, norfloxacin lethality was reduced. These data are consistent with superoxide dismutase normally promoting quinolone lethality, perhaps by stimulating formation of peroxide [93]. A deficiency in catalase/peroxidase (*katG*) also elevated the lethal activity of norfloxacin, a result that was expected because a buildup of peroxide should lead to accumulation of highly toxic hydroxyl radical [93].

Collins *et al*. also reported that bacteriotstatic concentrations of thiourea or 2,2'-bipyridyl, agents expected to reduce the level of hydroxyl radical, inhibit norfloxacin lethality [91]. That led to the conclusion that hydroxyl radical contributed to quinolone lethality [91]. Since inhibiting growth of *E. coli* is known to block norfloxacin lethality, thiourea and 2,2'-bipyridyl treatment was reinvestigated at subinhibitory concentrations. Even then the two agents interfered with norfloxacin-mediated killing [93]. Thus, ROS are very likely to play a role in quinolone-mediated lethality.

Since norfloxacin displays a complex behavior with respect to lethal action [63], we reinvestigated the role of ROS using oxolinic acid, which kills only by the chloramphenicol-sensitive pathway. Like chloramphenicol, thiourea plus 2,2'-bipyridyl almost completely blocks the lethal action of oxolinic acid [94]. But only chloramphenicol blocks chromosome fragmentation (X. Wang and X.Z., unpublished observation). Thus, the chromosome fragmentation step occurs before the ROS step. Apparently chromosome fragmentation caused by oxolinic acid can be repaired, which explains the ability of inhibitors of ROS to almost completely block cell death (in the next section we discuss a possible involvement of the Lon protease in repair). As expected, a surge of hydroxyl radical accumulation follows oxolinic acid treatment, and that surge is blocked by chloramphenicol (X. Wang and X. Z., unpublished results).

Lethal action of PD161144, a C-8-methoxy fluoroquinolone that kills *E. coli* by the subunit dissociation pathway, is affected little by treatment with chloramphenicol or thiourea plus 2,2'-bipyridyl or all three agents together (X. Wang and X.Z., unpublished observations). These data further distinguish the two lethal pathways, and they suggest that lethality from subunit dissociation is independent of ROS generation.

LON PROTEIN AND REPAIR OF QUINOLONE-MEDIATED LESIONS

Lon protease degrades abnormal proteins and proteins produced in excess [95]. By targeting regulatory proteins,

Lon influences a variety of physiological phenomena, including cell differentiation, sporulation, pathogenicity, and survival during starvation and anaerobic conditions. The Lon protein has ATPase activity, and part of the ATPase domain binds DNA. As a result, early studies identified Lon as a double-strand DNA binding protein [96]. Large DNA molecules stimulate both the ATPase and the protease activities of Lon, which led to speculation that Lon might bind chromosomal DNA adjacent to regulatory proteins where it could control their turnover [97]. However, several *in vitro* studies show that the interaction of bacterial Lon with large DNA molecules lacks nucleotide sequence-specificity [98]. Nevertheless, Lon remains a candidate for removal of proteins, such as topoisomerases, trapped on bacterial chromosomes.

We first noticed a role for Lon protease in chromosome maintenance when examining paradoxical survival of bacteria at very high concentrations of quinolone [99]. A deficiency of Lon protease eliminates paradoxical survival [100]. Plasmid-borne protease activity of Lon restores the paradoxical behavior of quinolones, while ATPase activity does not. These observations confirm that Lon is necessary for paradoxical survival and indicate that the protease activity is indispensable.

To determine whether Lon affects chromosomal breaks in cleaved complexes, an empirical viscometric assay was applied to lysates of cells treated with various concentrations of nalidixic acid. When SDS was added to cell lysates to unfold chromosomes and release broken DNA from cleaved complexes, viscosity of lysates paralleled the lethal effect of nalidixic acid, initially dropping as nalidixic acid killed cells and then rising as high drug concentrations protected from death. In the Lon-deficient mutant, cell lysate viscosity was low when cells were treated with the high drug concentrations that had been rendered lethal by the *lon* mutation. Thus, cleaved complexes paralleled bactericidal effects, including those influenced by Lon.

Lon may also recognize and help repair other forms of cleaved complexes. One involves the derivative of ciprofloxacin containing an N-bromoacetyl C-7 piperazinyl group (Cip-Br). We expected the bromo substituent to form cross-links with a nearby cysteine, and bacterial strains having a Gly-81 Cys substitution in GyrA were exceptionally susceptible to Cip-Br. Moreover, inhibition of DNA synthesis by the quinolone was not reversed by washing cells with drug-free medium (M.M. and A.M. unpublished observations). However, lack of reversal by Cip-Br was seen only in a Lon-deficient strain, as if preferential recognition and removal of Cip-Br-Cys complexes by Lon obscured the irreversibility of putative drug-gyrase crosslinking.

A third example of Lon-mediated protection from quinolone was observed following treatment of *E. coli* cells with chloramphenicol, a bacteriostatic agent. With wild-type cells, chloramphenicol blocks further killing by oxolinic acid, even when added an hour after the quinolone. However, in Lon-deficient strains, chloramphenicol fails to rapidly halt quinolone-mediated cell death. If chloramphenicol is added before quinolone, the absence of Lon has no effect. These data are consistent with Lon being involved in the repair of lethal lesions formed by quinolones, lesions whose formation is blocked by chloramphenicol. Whether Lon-mediated repair involves direct removal of the complexes or an indirect effect due to rapidly removing an unidentified lethal factor involved in fragmentation of DNA is not known.

Each example of Lon action on cleaved complexes involves a situation in which the complex may have an unusual structure (extra drug molecules bound at the high quinolone concentrations that allow paradoxical survival, cross-linked drug-gyrase complexes formed with Cip-Br, and lethal, rather than reversible complexes after prolonged quinolone treatment). A Lon deficiency has no observable effect on nalidixic acid-mediated killing in an otherwise wild-type strain at low to moderate concentrations [100]. Under these conditions, Lon-mediated repair may be unable to compete with ROS-mediated killing.

We next turn to quinolone resistance. For many years, the prevalence of resistance was low for most pathogens, and the absence of plasmid-borne resistance was touted as one of the virtues of the fluoroquinolones. Heavy medical and agricultural use has negated both statements.

MUTANT SELECTION WINDOW

Stepwise Selection of Resistance Mutants

As with many antimicrobials, resistance to fluoroquinolones is conferred by genetic variations that reduce intracellular drug concentration (e.g. activation of efflux pumps) and/or reduction of the affinity of the compound for its target. With some pathogens, a single mutation, either chromosomal or plasmid-borne, is insufficient for clinical resistance. In such cases, it is often the accumulation of multiple changes that lowers susceptibility enough to achieve resistance. If the initial drug concentration is low, non-target alleles will be selected, as seen with mycobacteria and *S. pneumoniae* [19, 101, 102]. If the initial concentration is moderately high, target mutations are selected [19, 103, 104, 102]. After the population acquires one mutation, a second emerges [105]. The order in which target and nontarget alleles arise probably depends on the incremental increase in quinolone concentration. Repeated cycles of fluoroquinolone challenge, punctuated by periodic outgrowth of pathogen populations, are expected to cause stepwise accumulation of mutations and therefore a wide variety of resistant mutants [14, 106].

The gradual accumulation of resistance alleles causes surveillance studies to underestimate the emergence of resistance, since strains can contain resistance mutations and still be considered clinically susceptible by MIC breakpoint criteria. Those mutations increase the propensity for attaining additional resistance determinants by raising the upper limit of the selection window (discussed below). Eventually strains accumulate enough mutations for MIC to exceed the resistance breakpoint. Dissemination of these resistant mutants can then cause a rapid increase in the prevalence of resistance, as has been observed with *S. aureus* [107, 108]. Consequently, resistance can appear to arise suddenly even though the early stages are intrinsically gradual. To see the early stages it is necessary to perform a population analysis in which large numbers of cells from a

culture are applied to antibiotic-containing agar plates and resistant colonies are counted [109]. The colonies that arise reflect the mutant subpopulations present in the culture.

Mutant Selection Window Hypothesis

In the late 1990s we noticed that the recovery of mycobacterial mutants from agar plates displays a characteristic response to fluoroquinolone concentration [102, 110]. At low concentrations, the drug has no effect on colony formation until MIC is approached; then colony recovery drops sharply as susceptible growth is blocked. As drug concentrations increase, a broad plateau is observed, since a variety of resistant mutant subpopulations can grow and form colonies at those levels of drug exposure. Eventually a high concentration is reached at which colony recovery drops sharply a second time. The second drop correlates with the MIC of the least susceptible first (single)-step mutant subpopulation. This value is designated as the mutant prevention concentration (MPC) because it severely limits the recovery of resistant mutants. At concentrations above MPC, bacterial growth requires the acquisition of two or more concurrent resistance mutations, which is a rare event. At low drug concentrations (slightly below MIC), selection pressure is greatly diminished because resistant mutants have no growth advantage over susceptible cells. Thus, the selective amplification of resistant mutants occurs in a drug concentration range that is above MIC but below MPC. This drug concentration range is called the mutant selection window.

Experimental Support for the Selection Window Hypothesis

Since the mutant selection window was derived from static measurements, either with agar plates [110] or with large volumes of liquid medium [111], it was important to determine whether the window also exists when drug concentrations fluctuate. Measurements with *in vitro* dynamic models show that the window can be observed with fluctuating antimicrobial concentrations for fluoroquinolones, vancomycin, and daptomycin [112-121]. It is also readily seen in rabbits infected with *S. aureus* and treated with levofloxacin [122]. With both *in vitro* and *in vivo* experiments, static data fit well with dynamic measurements.

The selection window hypothesis differs qualitatively from the conventional idea in which the danger zone for selection of resistant mutants lies below MIC [123] rather than between the MIC and MPC [124]. The two ideas make different predictions about the emergence of resistance. According to the conventional view, eradication of the susceptible population will suppress acquisition of resistance ("Dead bugs don't mutate" [125]). In contrast, the selection window hypothesis maintains that resistance can emerge even when the susceptible population is eliminated, because resistant mutants may exist in a bacterial population prior to the start of antibiotic treatment. Treatment then allows mutant enrichment and amplification. *In vitro* and animal studies described above support the window hypothesis, as does a small clinical trial [126]. In the clinical study, newly hospitalized tuberculosis patients were screened for nasal colonization by *S. aureus* and then treated for tuberculosis

using a protocol in which rifampicin was the only agent active with *S. aureus*. After several weeks, patients were again sampled for *S. aureus* nasal colonization. In 92% of the cases, *S. aureus* colonization was eliminated, which showed that the treatment was effective. The other 8% of the colonizing isolates became rifampicin resistant. DNA analyses indicated that the resistant isolates evolved from the original, susceptible ones rather than from re-infection with different strain types. Collectively, these are the results predicted by the window hypothesis for a situation in which drug concentration is inside the selection window during treatment (MPC for rifampicin resistance is very high [20], which placed therapeutic concentrations inside the selection window).

Lethal Action and Resistant Mutant Selection

One of the predictions of the selection window hypothesis is that the emergence of resistance can be restricted by keeping drug concentrations above the selection window. This strategy is based on blocking mutant growth. Lethal action is an added effect that directly reduces susceptible pathogen numbers. That should help shorten treatment times, which in turn should reduce costs, toxic side effects, and the chance that new resistance will develop. Removal of the major population of susceptible cells should also increase the probability that host defense systems will eliminate resistant mutants.

Lethal action has additional importance for fluoroquinolones having gyrase as their primary target because gyrase-mediated resistance is genetically recessive. A recessive resistance mutation is not phenotypically expressed until the resistant, mutant protein has replaced most of the sensitive, wild-type copies (*E. coli* contains more than a thousand gyrase molecules per cell and hundreds of cleaved complexes form on chromosomes [8, 70, 127]). Until that time, the mutants will still be killed by the quinolone. Consequently, compounds that are more lethal will be better at restricting the selection of newly formed resistant mutants. When topoisomerase IV is the main target, resistance is codominant [60]; consequently, resistance would be expressed soon after the mutation occurred. In this situation lethal action would not have as great an effect as when resistance is recessive. Recessive-dominant considerations may partly explain why the frequency for obtaining target mutants of *S. pneumoniae* is 1,000 times higher for fluoroquinolones whose primary target is topoisomerase IV rather than gyrase [19, 128].

Pharmacodynamics and the Selection Window

Some of the complexities of lethal action on the selection window can be bypassed by empirical PK/PD considerations, since they take into account both bacteriostatic and bactericidal activity. For antimicrobial pharmacodynamics, the efficacy of a compound is commonly related to two parameters, its potency against the bulk population of a particular pathogen, usually measured as MIC of the pathogen culture, and the concentration achieved at the site of infection. For the so-called concentration-dependent killers, such as fluoroquinolones, the two parameters are conventionally combined by dividing the area under the time-

concentration curve in a 24-hr period (AUC_{24}) by MIC. This pharmacodynamic index (AUC_{24}/MIC) correlates empirically with favorable patient and microbiological outcome [129, 130]. To extend this idea to restricting resistant subpopulation enrichment, MIC is replaced with MPC (the MIC of the least susceptible mutant subpopulation). Thus, a value of AUC_{24}/MPC can be determined experimentally to define the upper boundary of the mutant selection window; that value takes into account lethal activity of fluoroquinolones with resistant mutants [131, 132] and better defines *in vivo* window boundaries [122]. Consequently, treatment with lethal agents should require maintenance of fluoroquinolone concentrations above MPC long enough for killing to occur, but not throughout therapy, as would be the case for bacteriostatic agents. Experimentally, restricted amplification of resistant mutant subpopulations requires fluoroquinolone concentrations to be above MPC for only 20% of the dosing interval when *S. aureus* is treated with levofloxacin [122].

A general population-based approach has been developed to relate dose and patient outcome through measurements of AUC_{24}/MIC [123, 133, 134]. The idea can also be used to evaluate particular doses for their ability to restrict the emergence of resistance [132]. Briefly, an animal model of infection is used to determine a target value of AUC_{24}/MPC at which no resistance emerges. Then the ability of a given dose to attain the targeted AUC_{24}/MPC with a human population is estimated by 1) determining AUC_{24} for the given dose using a patient population, 2) determining pathogen MPC for the compound using isolates from the patient population to be treated, and 3) mathematically combining the population AUC_{24} and pathogen population MPC. Due to the pharmacokinetic diversity of patient populations and susceptibility diversity of bacterial isolates, the output is the fraction of the patient population that will reach the pharmacodynamic target using a particular dose. Widespread use of this method requires additional measurements of pathogen population MPC [135-137] since MIC cannot be reliably used to predict MPC [138, 139].

Practical Importance of the Window Hypothesis

A major feature of the hypothesis is that it reveals one reason why emergence of resistance is occurring: clinical treatments place drug concentrations inside the window for long periods of time. That facilitates mutant enrichment unless host defenses eliminate or block proliferation of mutant subpopulations. It also provides a general approach for slowing the emergence of resistance: keep drug concentrations above the window or use combination therapy. Applying this strategy is difficult because no existing antimicrobial has been developed using selection window principles. Moreover, restricting resistance generally requires higher doses than needed to cure most patients; consequently, toxic side effects become an issue. However, human pharmacokinetics for approved doses are known, and MPC has been measured for many drug-pathogen combinations. Consequently, compounds can be compared for their ability to restrict the emergence of resistance.

QUINOLONE-INDUCED QUINOLONE RESISTANCE

The mutant selection window addresses selective amplification and enrichment of resistant mutants, but it does not consider the important property of mutant induction: the quinolones induce the mutagenic SOS response. To examine the effect of quinolone structure on the recovery of resistant mutants during drug exposure, we applied *E. coli* to agar plates containing various compounds, and at daily intervals we counted the cumulative number of colonies (this assay had been used previously to assess the effect of various mutations on induction of resistance [140, 141]). With *E. coli,* colonies seen after one day of incubation estimate a baseline of mutants pre-existing in the test culture. The number then increases over the next week. The rate of mutant accumulation probably depends on a complex set of factors that includes the rate at which wild-type cells are killed and blockage of mutant growth (M.M., unpublished observations). A methoxy group at position C-8 is particularly restrictive, and a quinazoline-2,4-dione is much more effective than its cognate fluoroquinolone. Thus, a simple agar-plate assay is available to compare compounds for the ability to restrict the mutagenic effects of quinolones and related antimicrobials.

NEW QUINOLONE-LIKE MOLECULES HAVING ANTI-MUTANT ACTIVITY

Antimicrobial development conventionally involves identifying new derivatives that are active against resistant mutants already enriched by earlier derivatives of the class. This approach keeps the clinician one step ahead of the pathogen. Experience tells us, however, that many pathogens can easily make one mutational step. New compounds are likely to have a longer life span if they require pathogens to acquire two or more concurrent resistance mutations for growth in the presence of the antibiotic. If the mutant selection window is closed, *i.e.* if MIC = MPC, two mutational steps will be required for growth. Thus, a goal of quinolone development is to close the selection window. That can be accomplished in two general ways. One requires a compound to have very good activity against resistant mutant subpopulations; the other involves a single agent having two independent targets with similar susceptibility or two agents having independent targets. Examples using the two general approaches are described below.

Closing the Selection Window with a Single Agent Having a Single Target

MPC is the MIC of the least susceptible, first-step resistant mutant subpopulation. MPC may be difficult to measure with some compounds and some bacteria because large numbers of organisms must be tested (on the order of 10^{10}). As an initial screen, compounds can be tested for anti-mutant activity. This activity is defined as the MIC of a known resistant mutant divided by the MIC of an isogenic wild-type (susceptible) strain. When a battery of resistant mutants is examined, compounds can be compared for their ability to suppress the growth of mutants: compounds are sought that have $MIC_{mutant}/MIC_{wt} \leq 1$. Then it is necessary to

measure MPC, since the battery of existing mutants may not have accurately represented the least susceptible resistant mutant subpopulation. For example, the novel compound being tested could have switched targets from gyrase to some other enzyme. Such a compound might be effective against existing gyrase mutants, but it would not necessarily have a narrow selection window, which could allow resistance to readily emerge.

Ellsworth *et al.* showed that the conversion from a quinolone core structure to a 3-amino-quinazoline-2,4-dione structure afforded gyrase inhibitors that are active against known quinolone-resistant mutants of *E. coli*, *S. aureus*, and *S. pneumoniae* [142-146]. Structurally similar pyrido[1,2-c]pyrimidine-1,3-diones appear to possess comparable activities [147], and we recently described an optimized synthesis of the 1,3-dione core ring system [148]. As a test of the anti-mutant approach, we prepared and evaluated a series of 3-amino-8-methoxy-quinazoline-2,4-diones (Fig. **3**) against quinolone-resistant *E. coli* mutants. By varying dione structure at the N-3 and C-7 positions, we were able to identify derivatives that brought the ratio of mutant to wild-type MIC close to unity [149], thus showing that optimized quinazoline-2,4-diones drastically reduce the protective effects of quinolone-resistant mutations in *gyrA* and *gyrB* of *E. coli*. Structural changes that lowered the ratio of mutant to wild-type MIC also lowered the absolute MIC. Moreover, the most bacteriostatic 2,4-dione exhibited rapid lethality similar to the cognate fluoroquinolone when normalized to MIC to correct for drug uptake/efflux [149]. When population analysis was performed with *E. coli* to examine the ability of the most active 2,4-dione to restrict the selection of resistant mutants, mutants were selected over a much narrower concentration range for the most active 8-methoxy-2,4-dione tested than for the cognate fluoro-quinolone or for ciprofloxacin [149].
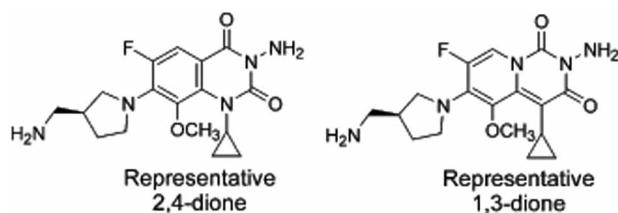

Fig. (3). Representative 3-amino-8-methoxy-quinazoline-2,4-dione and 5-methoxy-pyrido [1,2-c]pyrimidine-1,3-dione structures.

Compounds Active Against More than One Target

Some newer fluoroquinolones, des-fluoroquinolones, and other uniquely substituted derivatives target DNA gyrase and topoisomerase IV of certain organisms with near equipotent activity [150-154]. Analogs of other quinolone-like structural scaffolds, such as some heteroaryl isothiazolones (Fig. **4**), also show equipotent targeting of DNA gyrase and topoisomerase IV [155]. Consequently, a strain with a first-step mutation in one topoisomerase, DNA gyrase or topoisomerase IV, would still be inhibited by action at the other target. When the susceptibility of the two targets is equal, no selection window exists; for growth, a mutant must concurrently acquire mutations in both genes encoding the

targets. The benefit of dual-targeting quinolones to slow the selection of quinolone-resistant mutants is negated when these agents are employed against *M. tuberculosis* and other organisms that lack topoisomerase IV.

Another approach to creating dual targeting compounds is to link members of different antibacterial classes. Indeed, conjugating two antibiotics to give multi-targeting agents has been studied by many groups with many antibacterial agents [156, 157]. With quinolones, this approach is exemplified by covalently linked rifamycin-fluoroquinolone conjugates [158, 159] and oxazolidinone-quinolone conjugates [160]. Low mutation frequency and good activity against quino-lone-resistant gyrase mutants is observed because the conjugates are derived from antibacterial agents having different molecular targets. The same approach can be used with separate agents in combination therapies. Variations in potency of the individual components of conjugates and pharmacokinetic differences still allow emergence of resistance, albeit at a slower rate.

PLASMID-MEDIATED QUINOLONE RESISTANCE

Resistance carried by plasmids poses two threats to quinolone efficacy. First, plasmids can transmit resistance to multiple antimicrobials, thereby allowing quinolone resis-tance to be selected by use of other antibiotic classes and *vice versa*. Second, plasmids can introduce resistance determinants into a bacterial population at a much higher frequency than occurs through spontaneous mutation [161-163]. As a result, resistance is expected to emerge more rapidly from plasmid-borne resistance genes than from spontaneous mutations. The practical implication is that infections caused by pathogens containing drug-resistant plasmids need to be treated with elevated drug concentration even though the bulk population may be considered susceptible.

Three forms of plasmid-mediated quinolone resistance have been identified. The first and best studied involves Qnr [164], a protein that interferes with quinolone binding to gyrase and topoisomerase IV. The second type expresses the quinolone-acetylating Aac (6')-Ib-cr enzyme that inactivates compounds such as ciprofloxacin [165, 166]. The third involves an efflux pump encoded by *qepA* [167]. Of the three, Qnr appears to have the most activity, increasing MIC up to 250 fold (QepA increases MIC by 10 fold [168] and Aac (6')-Ib-cr by 4 fold [165]). Below we focus on Qnr.

Qnr was discovered in a strain of *Klebsiella pneumoniae* exhibiting resistance to fluoroquinolones and 13 other agents [164]. The discovery of related proteins (QnrB [169] and QnrS [170]) subsequently caused the original protein to be renamed QnrA. QnrA is a 218-amino-acid protein that belongs to a large protein family characterized by pentapep-tide repeats (the pentapeptide protein family includes roughly 500 members that display a wide variety of properties [171, 172]). Insight into how the gyrase-protecting subclass might act came from structural analysis of MfpA [173], a Qnr homologue found in mycobacteria [174]. When MfpA was expressed in *E. coli*, purified, and crystallized, its three-dimensional structure revealed that the protein dimer has size, shape, and electrostatic similarity to B-form DNA [173]. The protein appears to be a DNA mimic.
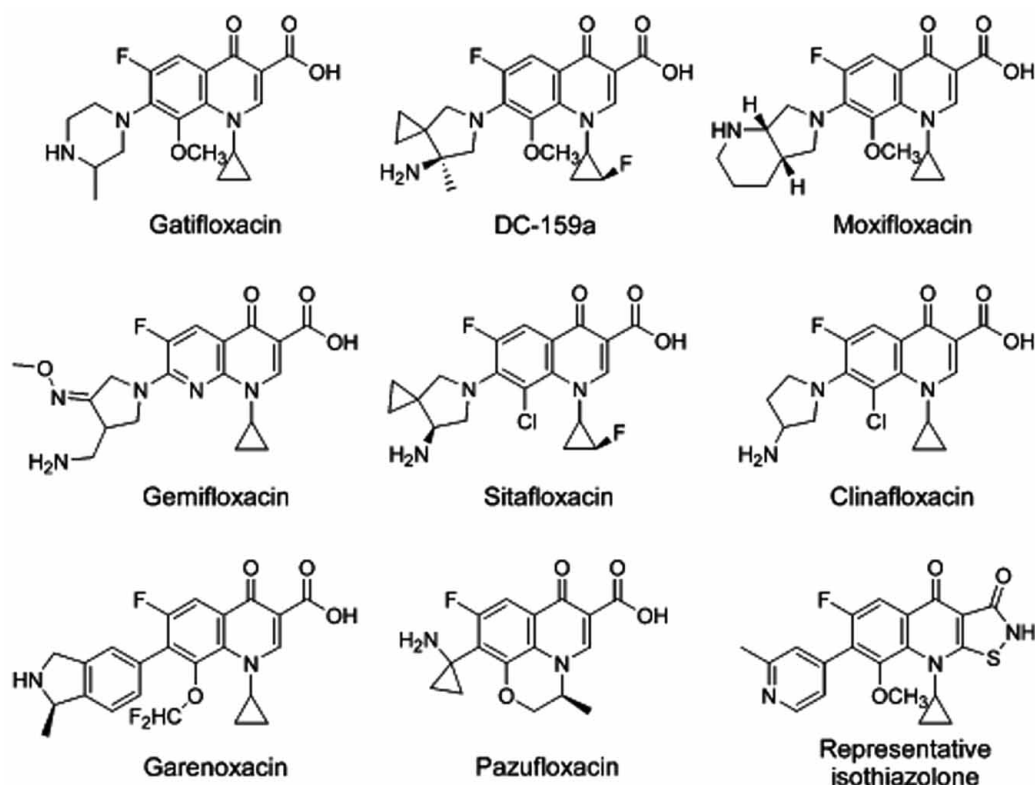
Fig. (4). Representative newer quinolone-class antibacterial agents found to be equipotent or near equipotent inhibitors of both DNA gyrase and topoisomerase IV. It is notable that each structure differs from early generation fluoroquinolones (e.g. ciprofloxacin and norfloxacin) by having a position-8 group other than simple aryl hydrogen.

The Qnr proteins lower quinolone binding to DNA complexes formed with gyrase or topoisomerase IV [175]. Binding of Qnr to the two enzymes appears to be specific rather than a general protein-binding property, and it does not require quinolone, DNA, or ATP [176]. Qnr also reverses quinolone-mediated inhibition of the supercoiling activity of gyrase [176]. Even a 1,000-fold excess of ciprofloxacin fails to overcome the Qnr-gyrase interaction; consequently, Qnr is likely to act by altering the DNA-binding properties of gyrase rather than by competitive binding to a quinolone interaction site [176]. Since quinolone resistance arises in a stepwise fashion, reduced susceptibility due to the presence of *qnr* is expected to be an important factor in the emergence of resistance, either by adding to the effect of an existing resistance allele to render a strain clinically resistant or by serving as an early step in the pathway to resistance. As expected, increased MPC has been reported with Qnr-containing bacteria [13].

Often plasmids having a QnrA determinant also carry genes that confer resistance to other anti-bacterials, such as aminoglycosides, β-lactams, chloramphenicol, and sulfonamides [177]. The presence of multiple antibiotic resistance genes on the same plasmid explains the frequent multidrug-resistant phenotype of Qnr-positive enterobacterial isolates. The fluoroquinolone-resistance plasmids are conjugative and carry both integrons and transposons [177]. They also have a broad host range: the plasmids have been obtained from a variety of enterobacteria including *Citrobacter freundii, C. koseri, Enterobacter aerogenes, E.*

amnigenus, E. cloacae, E. sakazakii, E. coli, Klebsiella oxytoca, K. pneumoniae, Providencia stuartii, Salmonella enterica, Serratia marcescens, Shigella dysen-teriae, and *S. flexneri* [178-181]. Moreover, Qnr-expressing plasmids are widely distributed with respect to geography [177]. For example, they have been isolated in Bangladesh [182], China [183-185], France [186], Germany [187], Korea [180], Japan [170], Taiwan [188], Thailand [189], Turkey [190], United Kingdom [191], and the United States [181, 192]. Thus, we expect *qnr* to cause serious resistance problems.

The genes responsible for the other two types of plasmid-borne resistance have not been studied as extensively as *qnr*. The quinolone-acetylating Aac (6')-Ib-cr enzyme inactivates compounds such as ciprofloxacin by placing an acetyl substituent on the unsubstituted nitrogen of the C-7 piperazinyl ring [165]. The enzyme also lowers susceptibility to norfloxacin, which has the same C-7 ring as ciprofloxacin. However, it has no effect on quinolones, such as enrofloxacin, pefloxacin, levofloxacin, and gemifloxacin, that lack an unsubstituted piperazinyl nitrogen [193]. So far, bacterial isolates carrying the *aac (6')-lb-cr* gene have been recovered from China [165, 185], France [194], the United States [15], and Uruguay [195] in a variety of Enterobacteriaceae such as *C. freundii, E. cloacae, E. coli,* and *K. pneumoniae* [195, 185]. The other plasmid-borne resistance factor, the QepA efflux pump, was first found in 2006 in a clinical isolate of *E. coli* from Japan [167]. MIC for hydrophilic fluoroquinolones, such as norfloxacin and ciprofloxacin, increases by 10 fold compared with plasmid-free counterparts [168]. So far,

the prevalence of QepA-mediated resistance in humans is low (0.3% among *E. coli* isolates collected from 140 Japanese hospitals between 2002 and 2006 [167]; 0.8% of ESBL-producing enterobacterial isolates collected in France during 2007 [196]).

CONCLUDING REMARKS

The quinolones continue to be an important class of antimicrobial agent. The reaction mechanism of the target enzymes is understood in considerable detail, and it is clear that formation of drug-enzyme-DNA complexes is the central event in quinolone action. However, our knowledge of these complexes is far from complete. For example, a crystal structure of the complex has been reported [33], but an additional structure may be required to explain what is likely to be multistep binding [197]. Release of DNA breaks from cleaved complexes and the resulting chromosome fragmentation continues to explain lethal action, although with the older quinolone derivatives it is clear that death ultimately arises from the accumulation of hydroxyl radicals. How the DNA breaks are released from protein-mediated constraint and how they promote a cascade of reactive oxygen species is unknown. The newer fluoroquinolones also cause an ROS surge, but chromosome fragmentation appears to kill faster than ROS.

Bacterial resistance to the quinolones is a growing problem [198-200]. Many aspects are now predictable within the framework of the mutant selection window hypothesis: continued use of dosing regimens that place drug concentrations inside the selection window for long periods of time will surely erode the usefulness of the compounds. Whether keeping concentrations above the window sufficiently restricts the emergence of resistance remains to be seen, especially since plasmid-borne resistance is becoming widespread. Assays are now available to screen new compounds for the ability to restrict mutant amplification, and new derivatives are emerging. Thus, the future remains bright for the quinolone class if the research developments are exploited judiciously.

ACKNOWLEDGEMENTS

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