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DNA Gyrase, Topoisomerase IV, and the 4-Quinolones

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INTRODUCTION

The 4-quinolones are antibacterial agents that have as targets two essential bacterial enzymes, DNA gyrase and DNA topoisomerase IV. Gyrase controls DNA supercoiling and relieves topological stress arising from the translocation of transcription and replication complexes along DNA; topoisomerase IV is a decatenating enzyme that resolves interlinked daughter chromosomes following DNA replication. Since both enzymes are required for cell growth and division, it is not surprising that the quinolones are bactericidal. However, these compounds do not simply eliminate topoisomerase function: trapping of gyrase and topoisomerase IV on DNA probably leads to the lethal release of double-strand DNA breaks.

For three decades, the quinolones have been used for a variety of physiological studies, serving as convenient inhibitors of DNA synthesis and as probes for the study of topoisomerase-DNA interactions. Early work was performed with the prototype compound, nalidixic acid (Fig. 1), which was replaced in the mid-1970s by a more active derivative, oxolinic acid. Exceptionally potent fluorinated quinolones, such as norfloxacin and ciprofloxacin, began to receive attention in the 1980s, largely because they were clinically effective. There are now thousands of derivatives.

In this review, we attempt to draw a diverse body of knowledge into a unified view of quinolone action. At the same time, we try to identify gaps in the knowledge of quinolone and topoisomerase physiology to guide future efforts at understanding topoisomerase-chromosome interactions. We hope that one of the practical outcomes will be new ways for reducing the rate at which pathogenic bacteria become resistant to the quinolones and related drugs. We begin by briefly sketching relevant features of gyrase and topoisomerase IV. Then we consider how the quinolones affect cell physiology, and we conclude by briefly comparing their effects with those of bacterial proteins that act in similar ways and with antitumor agents that attack eukaryotic topoisomerases.

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DNA GYRASE

DNA gyrase is the bacterial enzyme that introduces negative supercoils into DNA. The protein binds to DNA as a tetramer in which two A and two B subunits wrap DNA into a positive supercoil (reviewed in references 158 and 163). Then one region of duplex DNA is passed through another via DNA breakage and rejoining (Fig. 2). Binding of ATP to gyrase drives the supercoiling reaction, with ATP hydrolysis serving to reset the enzyme for a second round of catalysis. In the absence of ATP, gyrase removes negative supercoils from DNA (47, 189). Since the ratio of ATP to ADP determines the final level of supercoiling achieved (200), [ATP]/[ADP] is a key aspect of the supercoiling-relaxation relationship. This makes gyrase and supercoiling sensitive to changes in intracellular energetics, which are themselves sensitive to aspects of extracellular environment such as salt concentration (66, 72, 76) and oxygen tension (29, 71). Temperature (13, 43, 51, 53, 120, 121) and pH (78) also influence supercoiling, but it is not clear whether changes in these factors also alter [ATP]/[ADP] in a way that would explain the changes in supercoiling.

The two subunits of gyrase are encoded by *gyrA* and *gyrB*, which are located on the *Escherichia coli* genetic map at 48 and 83 min, respectively (in some bacteria the two genes are adjacent and close to the origin of replication [42, 94, 191]). Temperature-sensitive alleles in either gene reduce chromosomal supercoiling (186, 198) and block initiation of replication at high temperature (40, 90, 138). Supercoiling is also lowered by inhibitors of gyrase (33) and reduction-of-function alleles that arise in response to a deficiency of topoisomerase I, the enzyme that prevents excess supercoiling from accumulating (28, 46, 154, 162). These decreases in supercoiling raise gyrase expression, indicating that a homeostatic mechanism exists for controlling supercoiling (117). *E. coli* gyrase also responds to changes in DNA twist elicited by intercalating agents and by temperature changes within the normal growth range (36, 51). Thus, gyrase and probably a certain level of supercoiling are important for cell growth.

Gyrase plays at least four roles in chromosome function. As mentioned above, one role is to maintain a particular level of negative supercoiling, which activates the chromosome for all processes involving strand separation. Since the level of super-

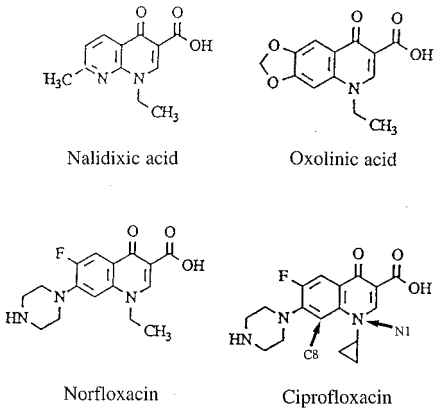

FIG. 1. Quinolone structures.

coiling appears to change in response to alterations in the extracellular environment, gyrase provides the genetic apparatus with a way to sense some types of environmental change. Gyrase also facilitates the movement of replication and transcription complexes through DNA by adding negative supercoils ahead of the complexes. The third function is the removal of knots from DNA (plasmid DNA contains six- to eightfold more knotted forms in cells carrying a reduction-of-function gyrase gene [177]). The fourth function is to help bend and fold DNA. The clearest example of this phenomenon is seen with bacteriophage Mu, which contains a strong gyrase binding site near the center of its genome. Inactivation of the site by deletion or insertion blocks the synapsis of prophage termini that precedes DNA replication (143–145). The bending activity of gyrase may also help explain its ability to suppress a growth defect due to the absence of HU, a small DNA-bending protein (111). Decatenation, a possible fifth activity of gyrase (185), is probably carried out primarily by topoisomerase IV, which is 100 times more active than gyrase at decatenating interlinked plasmid DNA (213; see below).

Evidence that gyrase is a target of the quinolones emerged soon after Gellert et al. discovered the enzyme (48). Nalidixic and oxolinic acids inhibited the supercoiling activity of purified gyrase when extracted from wild-type cells but not when extracted from resistant *gyrA* (*nalA*) mutants (47, 189). This inhibitory action involves trapping a gyrase-DNA complex in which the DNA is broken (47, 189). Comparable complexes, identified through DNA fragmentation, were later found on chromosomes isolated from drug-treated cells (181). As expected, quinolone-resistant *gyrA* alleles prevent chromosome breakage due to quinolone treatment (181). Mutations affecting quinolone resistance were also mapped to *gyrB* (207, 209, 210). When a *gyrA* mutation was found to only partially block the lethal action of ciprofloxacin (96), several laboratories began to consider the possibility that the quinolones had a nongyrase target.

DNA TOPOISOMERASE IV

In 1990, Kato et al. (80) discovered a homolog of gyrase that they called topoisomerase IV. Like gyrase, topoisomerase IV is composed of four subunits, two each of the *parC* and *parE* gene products (80, 81, 147). In *E. coli* and *Salmonella typhimurium*, the two genes map at 65.3 min (82, 108). The product of the nearby *parF* gene may facilitate DNA-dependent membrane binding of topoisomerase IV (81), since the ParF protein is very hydrophobic.

Both gyrase and topoisomerase IV use a double-strand-passage mode of action (163). However, the enzymes differ in a fundamental way: gyrase wraps DNA around itself, while topoisomerase IV does not (148). Indeed, wrapping seems to be the principal difference between the enzymes, since removal of a portion of the gyrase A protein converts gyrase into an enzyme that has a strong decatenating activity, much like that of topoisomerase IV (77).

Wrapping favors intra- rather than intermolecular strand passage, giving gyrase a poor decatenating activity relative to its supercoiling and relaxing activities. In contrast, topoisomerase IV is expected to recognize DNA crossovers (213), much as eukaryotic topoisomerase II does (164, 165, 214). This helps explain why the decatenating activity of topoisomerase IV is greater than its relaxing activity (67).

The DNA-wrapping difference between gyrase and topoisomerase IV probably contributes to functional differences. The prominent feature of purified topoisomerase IV is its ability to remove catenanes created by bidirectional replication, a property that is quite weak in gyrase at moderate salt concentrations (64, 65, 146). It appears that topoisomerase IV is able to decatenate DNA before completion of a round of replication whereas gyrase seems to decatenate only after the round is finished. Mutant phenotypes indicate that decatenation of replicated daughter chromosomes is indeed a major role of topoisomerase IV. For example, a temperature-sensitive mutation in *parC* causes an accumulation of catenanes in

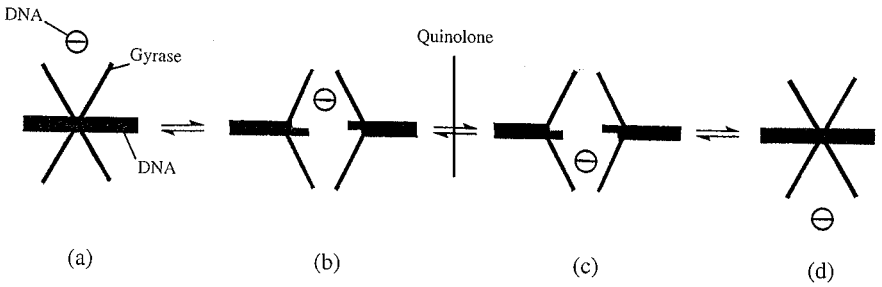

FIG. 2. Interruption of gyrase action by quinolones. (a) DNA gyrase and DNA before strand passage. Gyrase, represented schematically by thin crossed lines, binds DNA. (b) Opening of the DNA gate. Gyrase undergoes a conformational change, DNA is broken as a pair of staggered single-strand breaks, and another region of the same DNA molecule, shown in cross section, is brought close to the DNA gate. Quinolones trap gyrase at this stage. (c) Strand passage. The region of DNA shown in cross section passes through the DNA gate. (d) DNA gyrase and DNA after strand passage. The reactions are shown as reversible because gyrase can introduce and remove negative supercoils from DNA. The quinolones block both reactions. High [ATP]/[ADP] ratios drive the reaction to the right in the supercoiling direction, and coumarin antibiotics or low [ATP]/[ADP] ratios cause relaxation. Adapted from reference 23 with permission.

plasmid DNA (2, 83, 213) and prevents chromosomes from separating (82, 170, 187).

Before the discovery of topoisomerase IV, it was thought that gyrase might be the major decatenating activity. Gyrase mutants were known to have defects in chromosome partitioning (74, 82), and when a temperature-sensitive *gyrB* mutant was shifted to the nonpermissive temperature, most nucleoids appeared as distinct doublets when isolated and observed by fluorescence microscopy (185). The addition of purified gyrase to the nucleoid preparation converted a significant fraction of the doublets into singlets in a reaction blocked by oxolinic acid. Thus, gyrase appeared to be capable of resolving catenanes, as subsequently demonstrated with plasmid DNA by pulse-chase experiments (213). These data show that topoisomerase IV and gyrase have a redundant activity and explain why high-level expression of the gyrase subunits from plasmid-borne genes can suppress defects in topoisomerase IV although the reverse is not true (81). However, gyrase decatenates plasmid DNA at only 1% the rate observed for topoisomerase IV (213), and so normal gyrase levels do not bypass defects in the genes encoding topoisomerase IV. At present, the best explanation for the partition defect of temperature-sensitive gyrase mutations is the extensive relaxation that occurs (186), since that would interfere with the ability of topoisomerase IV to decatenate DNA (topoisomerase IV prefers a supercoiled substrate [148]).

The relaxing activity of topoisomerase IV can, under some conditions, suppress *topA* (topoisomerase I) defects that otherwise block growth (28). An example is seen when *parC* and *parE* are overexpressed from a plasmid in *E. coli* (80). Another may arise when the *parC-parE* region of the chromosome is duplicated (30, 156). Expression of *parC* and *parE* from a plasmid also suppresses a *topA* mutation in *Shigella flexneri*, restoring expression of the *vir* genes (115). Thus, topoisomerase IV provides redundancy for topoisomerase I. Whether topoisomerase IV normally contributes to chromosome relaxation has not been established.

Mechanistic similarities between gyrase and topoisomerase IV suggested that the latter might be a second target of the quinolones (81). It is now clear from a variety of assays, including measurement of DNA cleavage, decatenation, and relaxation, that purified topoisomerase IV is inhibited by quinolones (67, 83, 147). For the *E. coli* enzymes, inhibition of the decatenating activity of topoisomerase IV generally requires 15 to 50 times more quinolone than does inhibition of the supercoiling activity of gyrase (67, 81, 83, 147), although the difference was substantially smaller in one study (83).

Soon after the discovery of topoisomerase IV, it became clear that gyrase was not the only intracellular target of the quinolones: a *gyrA* (Nal^r) allele provided *E. coli* with only partial protection from ciprofloxacin (96), and a resistance marker called *flq* mapped outside the gyrase genes in *Staphylococcus aureus* (192). Within several years, topoisomerase IV was identified as a second target. With *E. coli*, the search began by the construction of norfloxacin-resistant strains by using sequential challenge to the drug (183). A *gyrA* mutation was identified, along with a mutation in *nfxB*, which maps at 19 min on the *E. coli* chromosome. Since these two mutations did not account for the high level of resistance, a search was made for a third allele. This turned up *nfxD*, which mapped at about 67 min on the *E. coli* genetic map (183), close to the position identified previously for *parC* and *parE* (80). It seemed reasonable that topoisomerase IV might be involved in high-level quinolone resistance; *nfxD* was subsequently shown to map in *parE* (12). A similar strategy of challenging existing *gyrA* mutants was used to obtain mutations conferring resistance to high levels of ciprofloxacin. These were mapped at or near *parC* by transduction (17). In another case, cells challenged by several successively higher levels of ciprofloxacin produced a *gyrA parC* mutant as determined by nucleotide sequence analysis (61). Meanwhile, nucleotide sequence homologies between *gyrA* and *parC*, plus the tendency of quinolone resistance to map in a particular region of *gyrA* (discussed in a subsequent section), made it possible to construct a *parC* mutation that raised the level of resistance in a quinolone-resistant *gyrA* mutant (83). Further support for the idea that topoisomerase IV is a target of fluoroquinolones in *E. coli* came from the finding that a *gyrA* (Nal^r) mutant contained a norfloxacin-sensitive decatenating activity and that plasmid catenanes accumulate when these cells are treated with norfloxacin (83). Since quinolone resistance alleles in *nfxD* (*parE*) or *parC* do not confer resistance by themselves (17, 61, 83, 183), topoisomerase IV must be a secondary target. A similar conclusion had been reached for *Neisseria gonorrhoeae* (6).

A slightly different scenario emerged from nucleotide sequence analysis of *S. aureus*. Clinical isolates that were resistant to a moderate level of ciprofloxacin each contained a mutation in a section of *parC* (*grlA*) called the quinolone resistance region. Isolates that were resistant to a high concentration of ciprofloxacin exhibited an additional mutation in *gyrA* (39). These observations suggested that topoisomerase IV, rather than gyrase, is the primary target of ciprofloxacin in *S. aureus*. Subsequent construction of laboratory strains by using stepwise challenges to increasing quinolone concentration confirmed that low-level resistance arises from mutation of *parC* and that high-level resistance is due to mutations of both *parC* and *gyrA* (38, 132). Resistance due to altered gyrase requires resistant topoisomerase IV for expression (132). The same phenomenon occurs in *Streptococcus pneumoniae* (126, 140), although sparfloxacin, a newer fluoroquinolone, appears to prefer gyrase as a target (141).

Gyrase and topoisomerase IV from both *S. aureus* and *E. coli* have been purified and studied for fluoroquinolone sensitivity (11). The supercoiling activity of *S. aureus* gyrase is at least 500-fold less sensitive to ciprofloxacin than is that of *E. coli* gyrase and about 6-fold less sensitive than is the decatenating activity of *S. aureus* topoisomerase IV. These data strongly support the assertion that topoisomerase IV is the primary target of ciprofloxacin in *S. aureus*. The decatenating activity of topoisomerase IV from *S. aureus* is only half as sensitive as that of topoisomerase IV from *E. coli*, so that the difference between the gyrase molecules accounts for most of the difference between the two organisms.

In summary, it is now quite clear that bacteria contain two topoisomerase targets of the fluoroquinolones. In some species, such as *E. coli* and *N. gonorrhoeae*, the primary target is gyrase; in other bacteria, such as *S. aureus* and *Streptococcus pneumoniae*, the primary target is generally topoisomerase IV. Since the two enzymes have different functions, it is likely that bacteria will differ in their response to the quinolones according to which enzyme is the primary target.

CLEAVED COMPLEXES

The central event in the interaction between the quinolones and gyrase or topoisomerase IV is formation of a quinolone-enzyme-DNA complex that contains broken DNA (47, 147, 181, 189). DNA fragmentation is readily explained in terms of gyrase-mediated strand passage (Fig. 2). As pointed out above, gyrase generates a pair of single-stranded breaks in a region of DNA wrapped around the enzyme. In a sense, a DNA gate is

opened through which another stretch of DNA can pass. Binding of ATP to gyrase provides directionality to strand passage, probably via a conformational change in gyrase. After strand passage, the gate closes and hydrolysis of ATP resets gyrase for another round. If a nonhydrolyzable analog rather than ATP is added to the reaction mixtures, only one round of strand passage occurs (188). The quinolones appear to trap the DNA-gyrase complex after DNA cleavage, at the open-gate stage (Fig. 2b). Since quinolone binding to gyrase-DNA complexes occurs even when DNA cleavage is prevented by a gyrase mutation (21), it is likely that "cleavable" complexes containing intact DNA can form. They may be converted to cleaved complexes upon gate opening, although this has not been demonstrated. Addition of a protein denaturant, such as sodium dodecyl sulfate, to quinolone-gyrase-DNA complexes releases DNA ends that are easily observed.

Hydroxyl radical and DNase I footprinting studies have been used to examine the fine structure of gyrase-DNA complexes (41, 84, 86, 123, 137, 157). In a hydroxyl radical study in which the major cleavage site in pBR322 was examined, gyrase, in the absence of ATP, protected about 120 bp of DNA, 50 bp on one side of the gyrase-dependent cleavage site and 70 bp on the other (137). A region of 13 bp adjacent to the cleavage site was more highly protected, with the site itself being quite accessible to hydroxyl radicals in the solvent. When a nonhydrolyzable analog of ATP, ADPNP, was used, a conformational change occurred in which additional DNA was wrapped around gyrase, the phase of hydroxyl radical cleavage maxima was shifted by 3 bp on one side of the gyrase cleavage site, and protein-DNA contacts were relaxed at the active site (137). Trapping of complexes by ciprofloxacin, norfloxacin, or oxolinic acid produced a pattern of protection very similar to that conferred by gyrase in the presence of ADPNP (137). Thus, footprinting experiments suggest that the quinolones and ADPNP allow gyrase to proceed to a similar step in its reaction with DNA, even though ATP is not required for binding of the quinolones to gyrase-DNA complexes (124, 174) or for the quinolones to block the relaxation of supercoiled DNA (47, 189).

It is uncertain whether binding of quinolones to gyrase-DNA complexes involves direct interaction with the DNA moiety, although it has been reported that the quinolones bind to purified DNA under conditions in which their interaction with gyrase alone is quite weak (171, 175). Saturable, cooperative binding of norfloxacin to supercoiled DNA appears to occur at the same drug concentration needed to inhibit the supercoiling activity of gyrase (175). The binding affinity to single-stranded DNA is three to five times higher. These conclusions about binding affinity, plus the finding that gyrase produces two staggered single-strand cuts, led to the idea that the 4-nucleotide single-stranded overlaps in the cleaved complexes might contribute to quinolone binding, perhaps through hydrogen bonding between quinolone molecules and bases of the DNA (172). Subsequent experiments, however, showed that binding of quinolones to gyrase-DNA complexes also occurred in the absence of DNA cleavage (21). Thus, the relevance of quinolone binding to DNA is unclear.

It has been proposed that the quinolones bind cooperatively to DNA, perhaps as a consequence of π-π stacking of planar quinolone rings (172, 173). Stacking does occur, at least in nalidixic acid crystals (1, 73), and examination of stereoisomers of ofloxacin reveals that the most potent form allows the closest stacking between the quinolone rings (59, 173). Additional cooperativity has been postulated to arise from hydrophobic tail-to-tail interactions between the N-1 substituents of quinolones (172, 173). Indeed, tail-to-tail interactions have been observed in crystals of nalidixic acid (1, 73). The distance between the N-1 atoms of interacting nalidixic acid molecules is very close to the distance between the N-1 atoms of two norfloxacin molecules covalently linked by four carbons. When the antigyrase activity of the four-carbon norfloxacin dimer was compared with that of dimers having linkers of different lengths, the four-carbon dimer proved to be the most active (172). Thus, the gyrase-DNA complex appears to accommodate two drug molecules tail-to-tail. It is unlikely, however, that this facilitates the trapping of one single-stranded break by one drug molecule and the second break by another inside cells, since double-strand cleavage in chromosomal DNA occurs as the accumulation of two independent single-strand events for each gyrase complex (181).

A different view of quinolone binding to DNA has emerged from studies with 2,2-quinobenzoxazine, a quinolone analog that inhibits eukaryotic topoisomerase II (37). Drug dimers form via magnesium ion bridges such that one monomer is proposed to intercalate into DNA while the other interacts externally with the phosphodiester backbone. The two quinolone molecules would interact very differently with DNA in this heterodimer model. In principle, it would be possible to improve quinolone efficacy by using two different structures if this model proves to be accurate.

Regardless of precisely how the quinolones bind to gyrase and DNA, one of the key findings is that complex formation is readily reversible. For example, removal of oxolinic acid from cells 15 min prior to lysis eliminates chromosome fragmentation (16). Even after lysis of drug-treated cells, DNA fragmentation can be reversed by mild heat treatment (60°C), as long as it precedes addition of the strong detergent used to denature gyrase (181). Thus it appears that gyrase can close the DNA gate once the quinolone is washed out. As emphasized below, the reversible nature of complex formation is important for understanding the physiological effects of the quinolones.

INHIBITION OF DNA SYNTHESIS AND BACTERIOSTASIS

It became clear soon after the discovery of nalidixic acid that the quinolones block DNA synthesis (27, 52). Gyrase was implicated when quinolone resistance mutations were attributed to alterations in *gyrA*. However, it seemed unlikely that quinolone action was due simply to the loss of gyrase activity, since bacteriophage T7 growth, which does not require gyrase activity, is blocked by nalidixic acid (90). Moreover, oxolinic acid had little effect on chromosomal DNA supercoiling under conditions in which the drug inhibited DNA synthesis by more than 95% (181). Thus, the concept emerged that the quinolones poison DNA synthesis (90).

The poison idea can be understood most easily by considering the effects of replication on DNA topology. As a replication fork moves in covalently closed circular DNA, duplex unwinding eventually removes the negative supercoils initially present. At this point, replication stops (106). The action of gyrase in front of a fork would allow replication to continue. In such a scenario, trapping gyrase in a quinolone-gyrase-DNA complex ahead of the fork would block fork movement. That would explain why oxolinic acid inhibits DNA synthesis rapidly, even when inhibition is only partial (181). The results of pulse-labelling experiments are consistent with gyrase being concentrated near forks: fragments of newly replicated chromosomal DNA are smaller than fragments of older DNA when cells are treated with oxolinic acid (31). Since gyrase is also distributed around the chromosome at roughly 100-kbp intervals (181), at low quinolone concentrations replication fork movement may

be blocked quickly by encountering a fork-associated complex or slowly by proceeding to a complex located at one of the widely distributed sites. Thus, the idea of fork blockage explains biphasic inhibition of DNA synthesis (35).

The lethal effects of the quinolones are frequently attributed to inhibition of DNA synthesis. Indeed, inhibition of DNA synthesis, formation of cleaved complexes, and inhibition of growth are correlated (18, 181). However, they are all reversible phenomena, while lethal action is not (27, 52). Moreover, the concentration of quinolone required to block DNA synthesis is lower than that required to kill cells (17, 52), and treatments with agents such as chloramphenicol, which block killing (see below), have little effect on inhibition of DNA synthesis (17, 27). Another argument derives from the observation that inhibition of DNA synthesis causes a *recBCD*-dependent breakdown of chromosomal DNA; if extensive, such breakdown should be lethal. However, DNA breakdown is not blocked by chloramphenicol or rifampin, whereas lethal effects are (99). In addition, breakdown is more extensive in a *recA* mutant than in a *recA recBC* mutant (201) whereas survival is much less likely when both recombination functions are defective (95). Thus, complex formation, inhibition of DNA synthesis, and DNA breakdown fail to provide satisfactory explanations for the lethal effects of the quinolones. As discussed in the next section, cell death probably arises from the DNA ends in the complexes being released from constraint by gyrase, creating the equivalent of double-strand DNA breaks.

Cleaved complexes also form with topoisomerase IV (147); in *E. coli*, they cause a slow inhibition of DNA synthesis that is apparent only when *gyrA* is resistant to quinolone action (83). Slow inhibition is consistent with topoisomerase IV-quinolone complexes forming at widely dispersed sites on the chromosome (17) and with topoisomerase IV activity not being located immediately ahead of replication forks. At present, there is no indication that topoisomerase IV is required for replication fork movement (170).

DNA BREAKS AND CELL DEATH

While seeking an explanation for quinolone-mediated cell death, we noticed that nucleoids isolated from cells treated with bactericidal concentrations of oxolinic acid sedimented more slowly than those from untreated cells (1,300S and 1,700S, respectively) (17). A similar sedimentation difference was known to arise from treatment of isolated nucleoids with low concentrations of DNase I or ethidium bromide, both of which relax negative supercoils present in chromosomal DNA (204). We then asked whether bactericidal concentrations of oxolinic acid would eliminate the negative supercoils present in chromosomal DNA (the moderate concentrations sufficient to block DNA synthesis do not [181]). Nucleoids were isolated from drug-treated cells, and the sedimentation coefficient was found to be nearly constant over a range of ethidium bromide concentrations that normally elicits the sharp sedimentation minimum characteristic of negatively supercoiled DNA (17). These data were most easily explained by the presence of free DNA ends, a conclusion further supported by the observation that the sedimentation rate of nucleoids from treated cells is sensitive to centrifugation speed in a way that is characteristic of large DNAs having ends. Since double-strand DNA breaks generated by other means are lethal (89), the liberation of DNA ends from quinolone-gyrase-DNA complexes accounts for the bactericidal action of the quinolones.

The idea of DNA end release was also tested by examining the effects of inhibitors of protein synthesis, since they block the lethal effects of nalidixic and oxolinic acids (17, 27) and should therefore eliminate the supercoil relaxation thought to arise as DNA ends become free from gyrase-mediated constraint. When cells were treated with chloramphenicol to inhibit protein synthesis, oxolinic acid failed to kill the cells or to eliminate the topological constraint needed for the maintenance of supercoils (17). Thus, it appears that a protein factor is involved in releasing DNA ends from the quinolone-gyrase-DNA complexes. Such a factor may turn over rapidly, since treatment of cells with chloramphenicol, even several hours after the addition of nalidixic acid, quickly blocks killing (22, 27). The factor, which has not been identified, is probably not a component of the SOS response, since a *lexA* mutation that blocks the SOS response has no effect on the rate at which cells are killed by nalidixic acid (95).

Although chloramphenicol and rifampin are very effective at blocking the lethal effects of nalidixic and oxolinic acids, their effect on the more potent fluoroquinolones is often only partial (17, 96). This suggested that the fluoroquinolones have an additional, chloramphenicol-insensitive mode of action (68). If the lethal effect of this second mode also arises from the release of DNA ends from quinolone-gyrase-DNA complexes, treatment of cells with ciprofloxacin should eliminate the ability of chromosomal DNA to restrain supercoils even in the presence of chloramphenicol. This is the case (17).

An explanation for the second lethal mode emerged from the observation that the quinolones stimulate a form of illegitimate recombination and deletion formation that is thought to arise from subunit dissociation-reassociation; i.e., quinolone molecules may be able to force gyrase-DNA complexes apart. Recent support for this idea has come from two types of genetic study. In one, the formation of specialized transducing phage by illegitimate recombination was stimulated 2 to 3 orders of magnitude by oxolinic acid (176). The major class of recombinant has short (3-bp) homologies that resemble the consensus quinolone-induced cleavage site found previously (107). Moreover, the effect is blocked by a mutation in *gyrA* that confers resistance to oxolinic acid but not by a mutation in *recA*. Thus, gyrase, rather than the RecA protein or components of the SOS response, is involved in this illegitimate recombination. In a second study, fluoroquinolones, at concentrations that barely prevented spore outgrowth, increased the recovery of deletion mutations in *Streptomyces ambofaciens* (197). Since oxolinic acid stimulates illegitimate recombination but has little lethal effect in the presence of chloramphenicol, recombination must be a much more sensitive assay than cell death for subunit dissociation. If so, fluoroquinolones should be far more potent than oxolinic acid at stimulating illegitimate recombination.

The data sketched above fit into a general scheme for intracellular quinolone action (Fig. 3). Complexes between gyrase and DNA are trapped by the quinolones in a reversible reaction that blocks DNA synthesis and cell growth (Fig. 3, pathway b). Cell death then arises in two ways. We propose that one lethal pathway involves removal of gyrase-drug complexes from DNA and liberation of lethal double-strand DNA breaks (pathway c). According to this idea, complex removal, which occurs with all quinolones, is blocked by inhibitors of either RNA or protein synthesis. We attribute a second mode to gyrase subunit dissociation while the enzyme is complexed to DNA (pathway d). This event is expected to release DNA ends, albeit with the gyrase subunits attached. We postulate that this second mode occurs when cells are treated with high concentrations of fluoroquinolones such as ciprofloxacin and that the lethal event is insensitive to inhibition of RNA or protein synthesis. Lethal effects arising from this chloramphen-

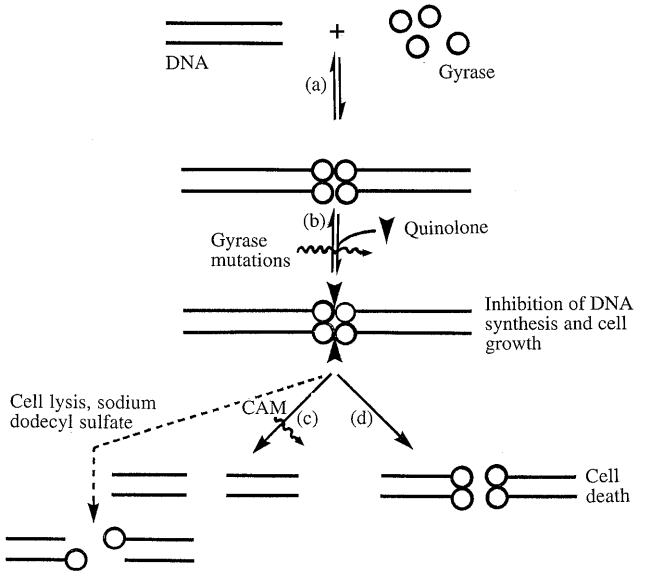

FIG. 3. Intracellular action of quinolones. (a) DNA gyrase and DNA interact to form a cleaved complex. (b) Quinolones trap the cleaved complex. Gyrase mutations prevent trapping by the quinolones. The trapped complexes block DNA synthesis and cell growth. (c) A putative trapped complex removal system releases lethal double-strand DNA breaks from the complexes. Chloramphenicol (CAM) or rifampin treatment of cells blocks this reaction. (d) Fluoroquinolones at high concentration stimulate gyrase subunit dissociation, which releases lethal double-strand breaks. The dotted line indicates release of staggered double-strand DNA breaks when cell lysates are treated with ionic detergents such as sodium dodecyl sulfate.

icol-insensitive mode of quinolone action become more prominent as the quinolone concentration increases (17, 68); perhaps drug stacking has a cooperative effect on gyrase subunit dissociation.

Much less can be said about cell death arising from quinolone-topoisomerase IV-DNA lesions. A straightforward hypothesis is that death arises from the same two modes seen with gyrase (Fig. 3). Support for a protein synthesis-dependent mode comes from the observation that killing of an *E. coli gyrA* (Nal^r) mutant is drastically reduced by rifampin (96) or chloramphenicol (17). Subunit dissociation with topoisomerase IV has not been observed; to see this mode, it may be necessary to examine *S. aureus*, an organism in which topoisomerase IV is more susceptible to the quinolones than gyrase.

Smith and colleagues have argued that the quinolones have three modes of lethal action (68, 97, 98, 101). By measuring cell survival, they found that nalidixic acid, norfloxacin, and ciprofloxacin fall into distinct categories when cell culture conditions are varied (summarized in Table 1). For example, all quinolones kill growing cells, norfloxacin and ciprofloxacin kill nongrowing cells suspended in saline, and only ciprofloxacin is lethal when protein synthesis is blocked. The first and third categories correspond to lethal action via pathways c and d, respectively, in Fig. 3. The behavior of the quinolones in the second, norfloxacin category can be rationalized if these compounds act by pathway c against both gyrase and topoisomerase IV while those in the nalidixic acid category only attack gyrase. Although there is little evidence to support this explanation, it has been reported that the addition of a *parC* resistance allele to a *gyrA* mutant of *N. gonorrhoeae* lowers its susceptibility to norfloxacin but not to nalidixic acid (26).

INDUCTION OF THE SOS RESPONSE

The quinolones are potent inducers of the SOS regulon, a set of genes involved in a variety of activities including DNA repair, recombination, and mutagenesis (56, 149, 150; for a review, see reference 199). Induction by the quinolones revolves around three proteins, RecA, RecBCD, and LexA. An early event is the generation of an inducing signal, which appears to consist of short oligonucleotides and/or single-stranded DNA. The RecBCD enzyme has properties that fit with its being the generator of the inducing signal (discussed below). The inducing nucleic acids activate RecA, which simultaneously or subsequently interacts with the LexA protein in such a way that autodigestion of LexA occurs (20, 105, 178). Since LexA is the repressor of the SOS regulon, its cleavage leads to high-level expression of the genes in the regulon. Activated RecA also causes cleavage of some phage repressors, explaining how quinolones induce lytic growth.

The nature and generation of the inducing signal is one of the least understood aspects of the induction process. Two non-mutually exclusive ideas have emerged. One maintains that short oligonucleotides are involved, largely because their delivery to permeabilized cells leads to induction (75, 134) and because single-stranded DNA is an inducer in vitro (20). A second idea is that single-stranded DNA produced by unwind-

TABLE 1. Responses of *E. coli* to three categories of quinolone

Biological response	Response to quinolone[a]			Reference(s)
	Nal	Nor	Cip	
Effects of culture conditions				
Growing cells killed	+	+	+	68
Rif/Cm[b]-blocked killing	+	+	±[c]	17, 68, 101
Nongrowing cells killed	−	+	+	68
Chromosome cleavage				
Chromosome breaks	+	ND	+	17
Cm-blocked chromosome breaks	+	ND	±	17
Effects of repair mutations				
Hypersensitivity of *recA* mutants	+	+	+	95, 114
Hypersensitivity of *recBC* mutants	+	ND	+	95, 114
Hypersensitivity of *lexA* (Ind^-) mutant	−	+	+	95
Effects of resistant gyrase (*gyrA*) mutation				
Growing cells killed	−	ND	±	96
Chromosome breaks	−	ND	±	17, 181
Lethal action blocked by Rif/Cm	NA	ND	+	17
Additional *parC* (Cip^r) allele blocks residual killing	NA	+	+	17, 83
Hypersensitivity of additional *recA* mutation	NA	ND	+	195
Hypersensitivity of additional *lexA* (Ind^-) mutation	NA	+	+	17
Hypersensitivity of additional *recA* (Ind^-) mutation	NA	ND	+	194

[a] Abbreviations: Nal, nalidixic acid; Nor, norfloxacin; Cip, ciprofloxacin; NA, not applicable because blocked by *gyrA* resistance allele; ND, not determined. In some experiments listed in the nalidixic acid category, oxolinic acid was used.
[b] Abbreviations: Rif, rifampin; Cm, chloramphenicol.
[c] ±, partial effect or reduced number.

ing of duplex DNA induces the SOS response. Both could be generated by the RecBCD protein, since it unwinds and degrades DNA. This enzyme, which is required for induction by nalidixic acid (104, 116, 179), displays four activities: duplex DNA unwinding, exonucleolytic degradation of duplex DNA, exonucleolytic degradation of single-stranded DNA, and endonucleolytic degradation of single-stranded DNA (for reviews, see references 128, 130, and 180). It has long been suspected that at least one of the nuclease activities is involved in induction, since suppressor mutations in *sbcA*, which restore nalidixic acid-mediated SOS inducibility to *recB* and *recC* mutants (79), lead to induction of another nuclease, exonuclease VIII. Mutants with mutations in *recBCD* narrow the field slightly, since nalidixic acid still induces the SOS response in those that lack detectable double-stranded DNA nuclease activity; consequently, this nuclease activity is unlikely to be required for induction (4, 14). Other *recBC* mutations block induction of the SOS response but have no effect on the single-strand-specific nuclease (79, 92). Thus, this nuclease is not sufficient to induce the response, although it could contribute to it. The remaining activity, DNA unwinding, thus becomes a likely candidate for producing the single-stranded DNA-inducing signal, either alone or in conjunction with the single-stranded exonucleolytic activity (4, 14).

Since the major activities of the RecBCD enzyme require the presence of a double-stranded DNA end, another question is how the enzyme gains access to the circular chromosome. In general, it is thought that replication forks provide an access point, since the RecBCD protein has greater access to replicating than to nonreplicating bacteriophage lambda DNA (184). Indeed, several *recBCD*-dependent phenomena associated with single-stranded disruptions in DNA can be explained by RecBCD accessing the chromosome after a replication fork has passed over the disruption, which would effectively produce a double-strand break and replication fork collapse (93). If replication fork movement is blocked by quinolone-gyrase-DNA complexes before the DNA interruptions are reached, the weak single-strand-specific endonucleolytic activity of the RecBCD enzyme (50) could attack the single-strand gaps present on lagging strands. Then the RecBCD enzyme itself would collapse the fork as it loads on the DNA.

In principle, the RecBCD protein could also load onto chromosomes of quinolone-treated cells at the double-strand breaks created by removal of cleaved complexes (Fig. 3, pathway c). Indeed, levels of RecA induction are elevated by quinolone concentrations that are 2- to 10-fold higher than those needed to block DNA synthesis or growth (56, 149, 151). These are the concentrations required to reveal double-strand breaks (17). However, the nuclease action of the RecBCD protein, as manifested by nalidixic acid-induced DNA breakdown, is affected little by treatment of cells with chloramphenicol (99), which blocks the removal of drug-gyrase-DNA complexes but not the inhibition of DNA synthesis (17). It is also unlikely that gyrase subunit dissociation (Fig. 3, pathway d) contributes substantially to RecBCD-mediated induction, since the level of maximal SOS induction is similar for quinolones that are thought to elicit subunit dissociation and for those that probably do not (149–151). Thus interruption of replication fork movement by complex formation remains the main focus for understanding early events in SOS induction by the quinolones.

Induction of the SOS response has two consequences. One is quinolone-induced mutagenesis, which requires the SOS response (149, 152). Induced mutagenesis could be very important if it contributes to the acquisition of quinolone-resistant mutations. The second consequence is enhanced survival in the presence of fluoroquinolones, an aspect developed in more detail below.

REPAIR OF LESIONS

Both RecA and RecBCD proteins are thought to participate in the repair of quinolone-induced damage because *recA* and *recBC* mutants are killed more quickly and extensively than are wild-type cells (114). Since a *recA recB* double mutant is more sensitive to nalidixic acid than is either single mutant (95), the *recA* and *recBC* functions do not completely overlap. A similar statement can be made for *recF* and *recBC* (114). Thus, there appear to be several pathways that contribute to the repair of quinolone-induced lesions. It is likely that these repair processes act after the formation of drug-gyrase (or topoisomerase IV)-DNA complexes, since the average distribution of chromosomal complexes generated by oxolinic acid is unaffected by the *recA56* mutation (30a) and since the ciprofloxacin concentration required to cause plasmid cleavage is the same for wild-type and *recA142* mutant cells (194).

RecA and RecBCD proteins play a role both in the SOS response and in recombination; consequently, experiments have been performed to estimate the relative contributions of the SOS response and recombination to cell survival. For some quinolones, such as nalidixic acid, survival depends largely on the recombination function of the RecA and RecBCD proteins, since the *lexA3* mutation, which blocks SOS induction but neither RecA- nor RecBCD-dependent recombination, has little effect on survival (79, 95, 114). The situation is slightly different for the fluoroquinolones norfloxacin and ciprofloxacin. Mutations that block SOS induction but not recombination (*recA430* and *lexA3*) increase the susceptibility of cells to fluoroquinolones (69). The qualitative differences between nalidixic acid and the fluoroquinolones probably reflect the nature of the damage created by the different types of compound. A common feature of norfloxacin and ciprofloxacin is their ability to attack topoisomerase IV, and indeed *recA430* and *lexA3* mutations do increase the ciprofloxacin susceptibility of *gyrA* (Nal^r) mutants (17, 194). Mutants carrying a *gyrA* (Nal^r) allele are also rendered more sensitive to ciprofloxacin by a *recA* null mutation than by the *lexA3* mutation (16). Thus, topoisomerase IV-mediated killing appears to be mitigated by both *recA*-dependent recombination and SOS induction.

Several other genes have also been implicated in the repair of lesions created by nalidixic acid. Mutations in *uvrB* cause a modest increase in sensitivity (113, 114), perhaps independent of the UvrABC nuclease, since *uvrA* mutants are not hypersensitive to the drug (54). *polA* mutants also show an increased sensitivity to nalidixic acid (203), suggesting that DNA polymerase I plays a repair role. Mutations in *uvrD* cause bacteria to become very sensitive; DNA helicase II may participate in late stages of repair (91). There has been little follow-up on these observations, and so it is not yet clear how these repair functions fit with the recombination and SOS activities discussed above.

QUINOLONE RESISTANCE DUE TO ALTERED GYRASE AND TOPOISOMERASE IV

Early studies of nalidixic acid mapped resistance to a gene that eventually became known as *gyrA* (47, 58, 189). When coexpressed with a sensitive *gyrA* allele, resistance is recessive (58, 131). This distinctive character probably derives from the large number of gyrase-DNA interactions that occur on the chromosome (5, 181), since some cleaved complexes will form in cells containing a mixture of sensitive and resistant gyrase

subunits. Intracellular removal of these complexes from the chromosome would then release double-strand DNA breaks, which are lethal even in small numbers (89). The recessive character of quinolone resistance has been used to attribute resistance to *gyrA* mutations in clinical isolates for a number of bacteria, including *E. coli*, *Klebsiella pneumoniae*, *Providencia stuartii*, *Pseudomonas aeruginosa*, and *Acinetobacter calcoaceticus* (62, 131). In these experiments, resistant cells were transformed with a plasmid carrying a wild-type *gyrA* allele from *E. coli*; resistant strains that acquired plasmid-borne quinolone susceptibility were inferred to be *gyrA* mutants.

For topoisomerase IV of *E. coli*, no clear dominance is seen when resistant and sensitive alleles are both present as single-copy genes. However, when either the sensitive or resistant allele is present in many copies, that allele is dominant (61, 83). In *S. aureus*, both *parC* and *parE* in the wild-type form must be overexpressed to confer sensitivity (206). The difference in dominance between gyrase and topoisomerase IV may reflect a situation where fewer topoisomerase IV cleavage complexes are trapped on the chromosome, as suggested by sedimentation studies in which DNA fragment sizes were measured following quinolone treatment (17).

Sequence analysis of DNA from many bacterial species shows that resistance mutations tend to alter amino acids near the putative active site in the GyrA protein (Tyr122 in *E. coli*). This region, extending between amino acids 67 and 106, is called the quinolone resistance-determining region (209, 211). A similar region is likely to exist in the ParC protein (6, 83). Within GyrA of *E. coli*, mutations of two codons, serine 83 and aspartic acid 87, give the greatest reduction in susceptibility (Table 2; for other bacterial species, the equivalent position may be offset by a few codons). Mutation of serine 83 to a hydrophobic amino acid generally confers more resistance than does mutation at position 87 (Table 2). When both sites are mutated, levels of resistance can be two- to threefold higher than when only one position is mutated (Table 2, *N. gonorrhoeae*).

As pointed out previously, gyrase is the primary target in *E. coli*, with *parC*-mediated resistance being detectable only in *gyrA* mutants and at high fluoroquinolone concentrations (17, 83). Accordingly, *gyrA parC* double mutants are less susceptible to fluoroquinolones than are *gyrA* single mutants (Table 2, *E. coli*, *H. influenzae*, and *N. gonorrhoeae*). Topoisomerase IV mutants do not by themselves confer resistance (17, 83). In *S. aureus*, topoisomerase IV is the primary target (38, 39, 132), apparently because *S. aureus* gyrase is much less susceptible to inhibition than is *E. coli* gyrase (11). Thus, *parC* mutations confer low-level resistance to ciprofloxacin, with an additional *gyrA* mutation increasing resistance (Table 2). Gyrase mutants do not by themselves confer resistance (132). The situation is similar in *Streptococcus pneumoniae* when ciprofloxacin is examined (126, 140, 142). However, sparfloxacin appears to attack *S. pneumoniae* gyrase more avidly, since *gyrA* mutations account for resistance of first-step mutants while *parC* mutations are seen only with second-step mutants (141). Thus, the quinolone structure can alter the target preference. While there are too many chemical differences between sparfloxacin and ciprofloxacin to attribute the target preference to a specific moiety, the results of other studies hint at the C-8 fluorine in sparfloxacin being important. In those studies, a C-8 chlorine provided DU6859a with higher activity against a quinolone-resistant gyrase (85).

Resistance mapping in *gyrB* also occurs, sometimes to moderately high quinolone concentrations (131, 210) and sometimes to low concentrations (153). Mutations that confer high levels of resistance map at specific sites, Asp426 and Lys447 of GyrB in *E. coli* (207). In *S. pneumoniae*, mutations arise in *parC* and *gyrA* before they arise in *gyrB* (140). The homologous gene for topoisomerase IV, *parE*, can also display resistance mutations (12). Recent crystal structure determinations of fragments of yeast topoisomerase II and GyrA protein (7, 112a) suggest that the quinolone resistance mutations in GyrA cluster around the active site for DNA cleavage, forming a quinolone-binding pocket. The quinolone resistance mutations in GyrB are likely to be at distant sites.

Examination of clinical isolates reinforces statements concerning the location of *gyrA* and *parC* mutations. For example, two studies with *E. coli gyrA* showed that mutation of serine to leucine or tryptophan at codon 83 occurred in 7 of 8 and 8 of 12 isolates, respectively; mutation of aspartic acid to valine or glycine at codon 87 occurred in the others (136, 139). Later studies focused on *parC* mutations, confirming that serine 80 and glutamic acid 84 tend to change to hydrophobic and positively charged amino acids, respectively (61, 195). In general, mutation of serine 83 of the GyrA protein is associated with moderate-level resistance, addition of one or two *parC* mutations correlates with increased resistance, three mutations (two *gyrA* and one *parC*) are associated with high-level resistance, and four mutations (two *gyrA* and two *parC*) are associated with very high levels of resistance (195). Ciprofloxacin resistance in *H. influenzae* and *N. gonorrhoeae* appears to follow the same pattern (26, 49). Thus, the mutation that confers the greatest resistance in laboratory experiments (serine to leucine at position 83 in *E. coli* GyrA protein or serine to phenylalanine at position 91 in *N. gonorrhoeae*) predominates in the clinic, and double mutations are associated with higher levels of resistance. Usually the double mutations are in *gyrA* and *parC*, but a clinical isolate of *Salmonella typhimurium* has been found in which both *gyrA* and *gyrB* are mutated (60). In *Staphylococcus aureus*, low-level resistance is associated with *parC* mutations and high-level resistance is associated with *gyrA parC* double mutations (39). In a large study with *S. aureus*, about one-third (149 of 451) of the isolates carried a recognizable *gyrA* mutation (190; *parC* was not examined). Of these mutants, almost 99% exhibited resistance, with two-thirds of the isolates exhibiting a serine-to-leucine mutation at codon 84 of GyrA and one-quarter having a glutamic acid-to-lysine mutation at codon 88. Taken together, these data fit well with studies of laboratory mutants even though we have ignored nontopoisomerase effects, such as efflux pumps, detoxification, and permeability factors.

Many mycobacteria appear to be naturally resistant to the quinolones: the GyrA position equivalent to *E. coli* codon 83 (codon 90) is the hydrophobic amino acid alanine (57), and gyrase purified from *Mycobacterium smegmatis* is less sensitive to inhibition by the quinolones than is gyrase from *E. coli* (122, 160). Mutation to a more hydrophobic amino acid (valine) renders mycobacteria even less susceptible (Table 2) (191, 205). The majority of clinical isolates of fluoroquinolone-resistant *M. tuberculosis* exhibit a mutation at codon 94 (205). This is the result expected if the wild-type alanine at codon 90 itself lowers susceptibility. Two mycobacterial exceptions, *M. fortuitum* and *M. aureum*, have a serine at position 90, making them more susceptible to ofloxacin than *M. smegmatis* and *M. kansasii* by a factor of 4 and more susceptible than *M. bovis* BCG and *M. tuberculosis* by a factor of 8 (57). So far, this phenomenon of natural gyrase-mediated resistance has not been observed in other groups of bacteria.

Fluoroquinolone resistance can be a major clinical problem with bacteria such as *P. aeruginosa*, *S. aureus*, and *M. tuberculosis*. Therefore, efforts are under way to find more effective derivatives. One approach has been to determine the bacteri-

TABLE 2. Effect of laboratory-generated *gyrA* and *parC* mutations on fluoroquinolone resistance

Organism	Mutation[a]		Relative resistance[b]	Reference(s)
	gyrA	*parC*		
Aeromonas salmonicida	S83I		40[c]	135
Escherichia coli	Q106H		4	209
	A67S		4	209
	G81C		8	209
	A84P		8	209
	D87N		16	209
	S83W		32	209
	S83L		32	34, 209
	S83L		10[d]	83
	S83L	S80L	60[d]	83
	S83L	E84K	100[d]	83
	S83L + D87G	S80I	>4,000	61
Haemophilus influenzae	D88N		8	49
	D88Y	S84I	20	49
	S84L	S84I	40	49
	S84Y + D88N	E88K	320	49
Mycobacterium bovis BCG	A90V		>3	191
	D94N		>3, 30	34, 191
Mycobacterium smegmatis	A91V		8	159
	D95G		16, 64	159
	A91V + D95G		32, 64	159
Mycobacterium tuberculosis	A90V		>3, 8	3, 191
	D94N		32	3
	G88C		>3	191
Neisseria gonorrhoeae	S91F		125	6
	S91F + D95N		250	6
	S91F + D95N	S88P	2,000	6
	S91F + D95N	S88P + E91K	8,000	6
Salmonella typhimurium	G81S		1	161
	A119V		14	55
	D87N		32	161
	D87Y		64	161
	S83F		64	161
	S83Y		64	161
	A67P + G81S		64	161
Shigella dysenteriae	S83L		8	155
Staphylococcus aureus		S80F	4	132
		S80Y[f]	4[e]	38
		E84K[f]	4[e]	38
		E84K[g,h]	8[e]	38
		S80Y[g,h]	8[e]	38
		E84K[g,h]	8[e]	38
	E88K	S80Y[g,i]	128[e]	38
	S84L	S80Y[h,j]	256[e]	38
	S84L	E84L[h,j]	256[e]	38
		S80F	4	206
		E84K	4	206
		S80F + E84K[k]	4	206
	S84L	S80F + E84K[k]	200	206
	S84L		1	132, 206
Streptococcus pneumoniae		K137N	2	126
		D83Y	4	126
		S79Y	4–16	126, 140
		A84T	8	140
	S83Y	D83T	16	126
	S83Y	S79Y	32–64	126, 140
	S83F	S79F + K137N	32	126
	E87K	S79Y	64	140

[a] Abbreviations represent the wild-type amino acid (in single-letter code), position number, and mutant amino acid.
[b] Numbers indicate the ciprofloxacin MIC for mutant cells relative to the MIC observed for wild-type cells.
[c] MIC obtained with flumequine rather than ciprofloxacin.
[d] MIC obtained with norfloxacin rather than ciprofloxacin.
[e] MIC obtained with sparfloxacin rather than ciprofloxacin.
[f] First-step mutant.
[g] Second-step mutant.
[h] Mutant exhibits reduced accumulation of norfloxacin.
[i] Mutant exhibits intermediate level of norfloxacin accumulation.
[j] Third-step mutant.
[k] Expressed from a plasmid.

cidal activities for large numbers of compounds by using *M. avium* and then use artificial intelligence systems to identify regions of the molecules that contribute to activity. The combination of a cyclopropyl group at the N-1 position with a fluorine or alkoxyl moiety at the C-8 position emerged as particularly potent (87), a finding that was later extended to many bacterial species (19). Since fluoroquinolones carrying a halogen substituent at the C-8 position tend to have side effects, a parallel effort with alkoxyl derivatives was carried out. Some C-8 methoxyl derivatives exhibited good bacteriostatic activity against a variety of bacterial species while also being less cytotoxic (167).

At about the same time, it became apparent that C-8 substituents might make fluoroquinolones especially effective against gyrase mutants. For example, a compound called DU6859a is 8- to 16-fold more effective than ciprofloxacin at blocking the growth of fluoroquinolone-resistant clinical isolates of *P. aeruginosa* (85). DU6859a contains a chlorine substituent at C-8, and removal of the chlorine correlates with a three- to eightfold reduction in the ability to block supercoiling by gyrase purified from resistant isolates and to block the growth of the isolates. In another example, we noticed that sparfloxacin, which has a fluorine attached to C-8, is about twice as effective as ciprofloxacin, which lacks that fluorine, against *gyrA* mutants of *M. tuberculosis* relative to a comparable sensitive strain (205). When we tested C-8 methoxyl fluoroquinolones against gyrase mutants of *E. coli*, we were surprised to find that the compounds were particularly lethal (unpublished observations). Since very lethal quinolones are less likely to allow mutations induced by the SOS response to be fixed in the population, they should be superior antibacterial agents. We tested this idea and found that resistant mutants did not accumulate when wild-type cells were challenged with a C-8 methoxyl fluoroquinolone (under the same conditions, control compounds lacking the C-8 methoxyl group allowed more than 1,000 mutants to be obtained [unpublished observations]). Thus, preexisting mutants can be used to identify a new generation of fluoroquinolones that requires wild-type strains to acquire more than one mutation before exhibiting resistance, an event expected to be exceedingly rare when multiple mutations are required.

QUINOLONE-LIKE ACTION BY PROTEINS

Some large plasmids ensure their persistence in bacterial populations by killing plasmid-free cells with proteins that attack gyrase in ways that resemble quinolone action. To protect plasmid-carrying cells, the plasmids also express proteins that neutralize the effects of the cytotoxic proteins. One of the toxic proteins, microcin B17, is a glycine-rich peptide with a molecular mass of about 3.2 kDa (25, 196). Genes involved in its production and secretion, as well as in immunity to its action, are found in large, single-copy *E. coli* plasmids such as pMccB17, pColX-W7, and pColX-CA23 (45, 168, 169). Several lines of evidence indicate that microcin B17 traps gyrase on DNA in cleaved complexes similar to those described above for the quinolones. For example, addition of protein denaturants following microcin B17 treatment releases chromosomal DNA breaks, many of which are located at or near sites seen when gyrase is trapped by oxolinic acid (196). Moreover, DNA synthesis is blocked rapidly by microcin B17 (90% in 20 min) (63), and resistance mutations, which map in *gyrB*, eliminate the recovery of broken DNA from microcin B17-treated cells and from extracts treated with the toxic protein (196). As expected, mutations in the *recA* and *recBC* genes drastically reduce cell survival following microcin B17 treatment (63). Microcin B17 and the quinolones (nalidixic acid) are also similar with respect to induction of the SOS response. In both cases, the *recA*, *lexA*, and *recBCD* genes are required for induction, as is active DNA replication: induction is sharply reduced in a temperature-sensitive *dnaA* mutant following depletion of replication forks by a shift to 42°C (63).

A major difference between microcin B17 and nalidixic acid is the irreversible nature of microcin B17 action (63). It has also been noted that mutations in *lexA* make cells hypersensitive to microcin B17 (63) whereas this is not the case for nalidixic acid (95). Whether the cytotoxic effect of microcin B17 is blocked by chloramphenicol, a feature characteristic of nalidixic acid, is not known. If it is, the lethal effect of microcin B17 probably arises from removal of complexes from DNA, as seen with the quinolones.

A gyrase-poisoning activity is also expressed by the F plasmid (119). Its cytotoxic protein, CcdB (LetD), kills cells and induces the SOS response unless CcdA (LetA), another product of F, blocks CcdB action (8–10, 182). As expected of an agent that interferes with gyrase action, overproduction of CcdB causes DNA relaxation (110). To explain why plasmid-free daughter cells are killed, it has been suggested that the half-life of CcdA is shorter than that of CcdB (9). CcdB is thought to trap gyrase on DNA as cleaved complexes, since broken plasmid DNA is recovered from CcdA-depleted cells following lysis with protein denaturants (9, 10). CcdA can reverse DNA cleavage and inactivation of gyrase activity even after CcdB has formed a complex with gyrase and DNA in vitro (10, 109); thus CcdB action must be reversible. Dilution of plasmid, and presumably depletion of CcdA, also leads to induction of the SOS response in a *recA*-, *lexA*-, and *recBCD*-dependent manner (4, 182).

Resistance to CcdB maps in *gyrA*, establishing an intracellular involvement of gyrase in the response. One gyrase mutation (9), conversion of Arg462 to Cys in the GyrA protein, is recessive, lies far outside the quinolone resistance region, and confers no resistance to nalidixic acid. Another mutation (118), conversion of Gly214 to Glu in the GyrA protein, also maps outside the quinolone resistance region, but it is transdominant. As with resistance to the quinolones and microcin B17, the CcdB-resistant *gyrA* mutation blocks double-strand DNA cleavage and induction of the SOS response following depletion of CcdA (9). A search for other host genes involved in the CcdB-GyrA interaction has implicated *csrA* as a negative regulator whose activity is overcome by *tldD*, *pmbA*, and *groE* (127). The *pmbA* gene also facilitates microcin B17 production (166). It has been suggested that this set of proteins plays a role in gyrase regulation (127).

Still another way to get quinolone-like effects is through gyrase mutations. For example, the *S. typhimurium* conditional lethal *gyrA208* mutation induces the SOS response in a *recB*-dependent way following a shift to the restrictive temperature (44). The temperature shift also triggers massive *recB*-dependent chromosomal degradation if *recA* is defective. This phenomenon is similar to the "reckless" death that arises from nalidixic acid treatment of *recA*-deficient cells (201). Apparently, the RecA protein acts as an attenuator of the RecBCD nuclease (88), and in the absence of RecA the nuclease degrades the chromosome if given access. This lethal effect of *gyrA208* is completely blocked by inhibition of protein synthesis (44). Such a finding could be explained by an induced system being involved in removing gyrase from DNA and exposing DNA breaks, since breaks might allow the RecBCD protein access to the chromosome. However, neither cleaved complexes nor rapid inhibition of DNA synthesis, which are char-

acteristic of the quinolones, was observed in the mutant when it was incubated at the restrictive temperature.

COMPARISON OF QUINOLONE AND ANTICANCER AGENT ACTION

Since all topoisomerases act by breaking at least one strand of DNA, entrapment of eukaryotic and prokaryotic topoisomerases should have similar features. Two types of antitumor agent trap the eukaryotic topoisomerases (for a review, see reference 15). Members of the camptothecin class form complexes with topoisomerase I, which has been shown to be the only target of the drug, with yeast as a model system (133). A defining feature of camptothecin action is the strong association of replication fork movement with the lethal action of the drug: camptothecin is particularly effective against cells undergoing replication, and its effect is eliminated when DNA synthesis is simultaneously blocked by aphidicolin (24). In vitro, addition of both topoisomerase I and camptothecin to a simian virus 40 replication system irreversibly traps advancing replication forks (193) and produces a linear, protein-bound replication product (70). These replication-associated double-strand breaks cannot be reversed by a mild thermal treatment, unlike the situation seen with single-strand breaks generated by topoisomerase I-camptothecin complexes formed away from replication forks (70). This peculiar behavior of replication complexes led to the idea that collision of a replication fork with a topoisomerase I-camptothecin-DNA complex could break the leading template strand prior to its replication. Unwinding and separation of the leading and lagging template strands would then reveal the equivalent of a double-stranded DNA break in the replicated leading strand (70). This idea explains why inhibition of DNA synthesis by camptothecin is only partially reversed by removal of the drug (103)—replication fork breakage is unlikely to be reversible. In contrast, inhibition of bacterial DNA synthesis by the quinolones is readily reversed (52), making it unlikely that the two drug types kill cells in the same way. Another difference between the two classes of compound is that camptothecin is a much more potent inhibitor of RNA synthesis than are the quinolones. Consequently, simultaneous treatment with another inhibitor of RNA synthesis, cordycepin, has little effect on the cytotoxicity of camptothecin (24). As pointed out in a previous section, inhibitors of bacterial RNA synthesis, such as rifampin, block the lethal action of quinolones such as nalidixic acid (27).

Members of the other class of eukaryotic topoisomerase inhibitor, represented by amsacrine (mAMSA) [4′-(9-acridinylamino)-methanesulfon-*m*-anisidide], attack topoisomerase II, a homolog of bacterial gyrase and topoisomerase IV. In contrast to camptothecin, mAMSA exerts its action throughout the cell cycle (24), indicating that interference with replication forks is not the only mode of mAMSA action. Indeed, inhibition of DNA synthesis provides only partial protection from the killing action of mAMSA (24). In this respect, mAMSA action may resemble that of the quinolones, since even in the absence of DNA replication the quinolones form complexes (17) that should be lethal (quinolone lethality has not been examined extensively when DNA synthesis has been blocked by other means).

The quinolones and mAMSA are also similar in their response to inhibition of RNA and protein synthesis. In bacteria, rifampin and chloramphenicol block the lethal action of nalidixic and oxolinic acids (17, 27), and in mammalian cells, inhibition of RNA synthesis, even when caused by camptothecin, partially protects from mAMSA-dependent cytotoxicity (24). This protection appears to occur with little effect on formation of topoisomerase II-DNA complexes, as assessed by a potassium-sodium dodecyl sulfate precipitation assay of protein-DNA complexes (24). A similar conclusion was reached for *E. coli* during treatment with oxolinic acid, when complex formation was assayed by DNA fragmentation (17). Thus eukaryotic cells may contain a system for removal of mAMSA-topoisomerase II-DNA complexes similar to the one we postulate for the lethal removal of quinolone-gyrase-DNA complexes in bacteria (Fig. 3, pathway c). It is also likely that a mechanism will exist for removal of topoisomerase I complexes; a good candidate has recently been found in yeast (208).

CONCLUDING REMARKS

Most of the data described in the previous sections fit into a scheme centered on the reversible formation of complexes among quinolone, gyrase/topoisomerase IV, and DNA. The complexes, which contain broken DNA, block DNA replication and bacterial growth without killing cells. We have argued that cell death arises from a subsequent release of DNA ends (17). For nalidixic and oxolinic acids, end release may come largely from removal of quinolone-gyrase complexes from DNA. For the fluoroquinolones of the ciprofloxacin class, we propose that DNA ends arise from both complex removal and dissociation of gyrase/topoisomerase IV subunits attached to broken DNA. Members of the norfloxacin class may be less able to separate the gyrase subunits. Newer fluoroquinolones, such as sparfloxacin and DU6859a, which carry C-8 substituents, probably fall in the ciprofloxacin class. They seem to attack gyrase more effectively (85).

Replication forks stall when reaching the complexes, and in an undefined manner they allow increased access of the RecBCD protein to the chromosome. That leads to DNA degradation, which is limited by interaction of RecBCD with Chi sites scattered throughout the chromosome (88, 129). Chi interactions inactivate the RecBCD nuclease and release a RecBC recombinase, which, along with RecA, participates in the repair of double-stranded DNA breaks (88, 129). The oligonucleotide products of nuclease action and/or single-stranded regions arising from unwinding by the RecBC protein stimulate the RecA coprotease, and subsequent cleavage of LexA induces the SOS regulon. An element of the SOS response provides partial protection against the lethal effects of fluoroquinolones but not against those of nalidixic acid. The identity of that element and its mode of action are yet to be discovered.

The existence of a system for removing drug-topoisomerase complexes from DNA is a key feature of the scenario sketched above. There is no direct support for a removal system; however, inhibitors of protein synthesis block the ability of oxolinic acid to kill cells and to break chromosomal DNA (17). Identifying genes involved in the putative removal system is one of the next tasks.

Some aspects of quinolone action fail to fit into simple schemes. One of the more puzzling observations is the inability of high concentrations of drug to kill cells as effectively as moderate concentrations (22). This paradoxical effect has been observed with a variety of bacterial species (22, 34, 102) and many different quinolones (97, 100, 101). The paradoxical effect cannot be due to induction of the SOS regulon, since the effect occurs in a *recA* mutant (95), nor can it arise by preventing the formation of quinolone-gyrase-DNA complexes, since they are present even at high quinolone concentrations (17, 181). Inhibition of RNA synthesis, which occurs at high quinolone concentrations (112, 202), might interfere with lethal removal of complexes containing nalidixic acid and thereby

create the paradoxical effect. However, inhibition of RNA and protein synthesis is not a satisfactory explanation for the behavior of fluoroquinolones such as ciprofloxacin, since at high concentrations these agents kill cells predominantly by a mode that is not blocked by inhibition of protein synthesis (17). Moreover, we have found a situation in which the paradoxical effect is seen even in the presence of chloramphenicol (unpublished observations). Thus, the loss of lethality at high quinolone concentrations remains unexplained.

Another mysterious observation concerns quinolone action under anaerobic conditions. With *E. coli*, complex formation appears to be similar to that seen in the presence of oxygen, with the number of complexes formed on the chromosome actually being slightly higher under anaerobic conditions (32). However, for at least some fluoroquinolones, cells are not killed in the absence of oxygen (97, 125). With *S. aureus*, killing is delayed rather than eliminated (212). The difference between *E. coli* and *S. aureus* may reflect differences in whether gyrase or topoisomerase IV is the primary target, but additional studies are required to sort out this oxygen-related phenomenon.

From a clinical perspective, the main problem with the quinolones is the accumulation of resistance mutations by target organisms. The recent demonstration that bacteria contain two targets for the fluoroquinolones raises the hope that new quinolones can be found that will effectively attack both targets and thus drastically reduce the probability of development of clinical resistance. Complex formation is likely to be an important step in defining drug potency; therefore, growth inhibition assays and measurement of cell lysate viscosity for DNA breaks (34) should be suitable for screening compounds. These simple assays make it feasible to carry out studies even with organisms that are difficult to study, such as the mycobacteria (205). However, our argument that complex formation and cell death are distinct events (Fig. 3) raises the possibility that some fluoroquinolones are surprisingly lethal. Indeed, preliminary studies show this to be the case for some C-8 methoxyl derivatives (unpublished observations). Therefore, we are optimistic about being able to find new, more effective quinolones.

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