



Molecular mechanisms of fluoroquinolone resistance

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Received: December 17, 2002 Accepted: December 30, 2002

Fluoroquinolones have a broad spectrum of activity for complicated urinary tract infections, gastrointestinal infections, respiratory tract infections, sexually transmitted diseases, and chronic osteomyelitis. Since fluoroquinolones are excellent antibiotics for a number of clinical indications, their consumption has increased rapidly, both in human medicine and in food animals. Resistance to fluoroquinolones is chromosomal mediated, involving mutations either in the target genes including DNA gyrase (*gyrA* or *gyrB*) and topoisomerase IV (*parC* or *parE*), or in the regulatory factors controlling bacterial permeability or the efflux capacity of the bacteria. This review focuses on mechanisms of fluoroquinolone resistance, including known and proposed molecular mechanisms. This review also discusses the clinical impact of fluoroquinolone-resistant bacteria.

Key words: Efflux pump, fluoroquinolone resistance, gyrase, topoisomerase IV

In the early 1960s, the discovery of the naphthyridine agent, nalidixic acid, opened the door for a series of quinolones [1,2]. Nalidixic acid was first introduced as a therapeutant for urinary tract infections caused by gram-negative organisms. The second generation of quinolone named fluoroquinolone (Fig. 1), such as ciprofloxacin and ofloxacin, resulted from fluorination, primarily at the position C6, has broad-spectrum activity for genitourinary, respiratory, gastrointestinal tracts, skin, and soft tissues infections caused by either gram-negative or gram-positive bacteria and sexually transmitted diseases [3,4].

Antimicrobial drug resistance is one of the factors causing treatment failure in not just nosocomial infections. To design new agents that provide effective therapy for infections caused by organisms resistant to older agents, we have to understand the mechanisms responsible for drug resistance in older agents. These mechanisms include reduction of drug accumulation, alteration of drug target, bypassing the drug-targeted enzyme, and inactivation of the drug. Resistance to fluoroquinolones is chromosomal mediated including mutations in the targets, overexpression of efflux pumps, and loss of porins.

Mechanism of Action

Cell wall synthesis, protein synthesis, nucleotide synthesis including RNA and DNA, and intermediary

metabolism are essential components of microbial metabolism and general targets of antimicrobial agents. The antibacterial activity of fluoroquinolones is inhibition of DNA replication. Fluoroquinolones have dual targets, topoisomerase II (DNA gyrase) and topoisomerase IV, which are related but distinct enzymes involved in DNA synthesis [5]. Fluoroquinolones stabilize DNA strand breaks created by DNA gyrase or topoisomerase IV by binding to the enzyme-DNA complex. These ternary complexes consisting of drug, enzyme, and DNA block the progress of the replication fork.

DNA gyrase and topoisomerase IV share a significant degree of homology at the protein level. Both enzymes have a tetrameric A_2B_2 structure [6,7]. The DNA gyrase in *Escherichia coli* consists of 2 GyrA and 2 GyrB subunits encoded by the *gyrA* and *gyrB* genes, respectively. The topoisomerase IV in *E. coli* consists of 2 ParC and 2 ParE subunits encoded by the *parC* and *parE* genes, respectively. The *gyrA* gene shares 36% identity and 60% similarity with *parC* at the amino acid level. The *gyrB* and *parE* genes have 42% identity and 62% similarity [8-10].

DNA gyrase has 2 functions: (1) to remove the positive supercoils during DNA replication, and (2) to introduce negative supercoils (one supercoil for 15-20 turns of the DNA helix) in the presence of ATP so that the DNA molecule can be packed into the cell [11,12]. The ATP binding domain is located in the N-terminal half of GyrB [9]. The DNA binding, DNA breakage, and interaction between A and B subunits are located in GyrA. The Tyr-122 residue in the N-terminal of GyrA participates in the breakage-reunion reaction with DNA

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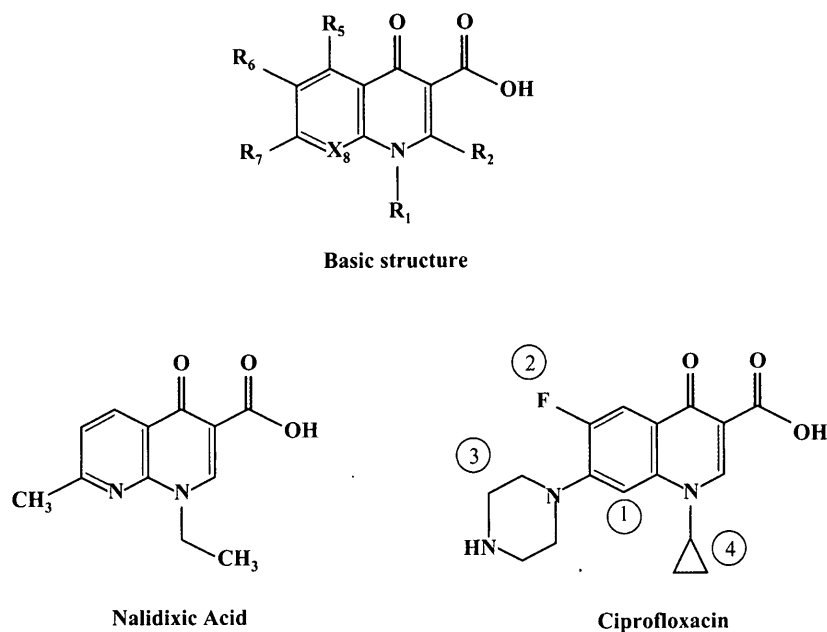


Fig. 1. The pharmacophore change from nalidixic acid to ciprofloxacin. ①Modification of naphthyridone into quinolone reduces plasma protein binding. ②Addition of the fluorine atom was shown to increase quinolone activity against DNA gyrase and facilitate penetration into the cell. ③Introduction of a piperazine results in a longer half-life. ④Replacement of the N-1 ethyl group by a cyclopropyl group can enhance potency against gram-positive and gram-negative bacteria.

and is also considered to be within the active site of the gyrase enzyme [9,12].

Unlike gyrase, topoisomerase IV does not wrap DNA around itself. Topoisomerase IV decatenates DNA before completion of a round of replication, whereas gyrase decatenates only after one round of replication [13]. The role of topoisomerase IV is to separate 2 linked DNA molecules [14,15]. Both gyrase and topoisomerase IV are essential for cell growth. Thus, they represent 2 potential lethal targets for quinolones.

Mechanisms of Fluoroquinolone Resistance

The antimicrobial agent has to enter the cells, find its target, disrupt the cellular function of cells, and eliminate the infecting organisms to establish a therapeutic success. There are many mechanisms, including plasmid or chromosomal mediated, contributing to a drug-resistant phenotype in infecting organisms. The potential mechanisms are: (1) reduction of drug accumulation including preventing the import of drug into the cell and activating the efflux of drug from the cell; (2) alteration of drug target by either mutating the target of drug, overexpressing the target, or bypassing the drug-targeted enzyme by changing other enzymes in the same enzymatic pathway; and (3)

inactivation of the drug by modifying or degrading the drugs. A drug-resistant organism has to employ at least one of these molecular mechanisms.

Target changes by alterations of DNA gyrase and/or topoisomerase IV, and reduced intracellular drug accumulation by reducing drug permeability and/or increasing efflux activity, are the major mechanisms mediating fluoroquinolone resistance (Fig. 2) [5]. Table 1 summarizes the mechanisms of fluoroquinolone resistance in bacteria. The evolution of resistance to fluoroquinolones arises in a stepwise fashion through the accumulation of spontaneous mutations in chromosomal genes [16-18]. As the first step, mutations in target genes are common, if not universal [5]. Earlier observation of plasmid-mediated resistance has not yet been confirmed [19]. Mutations in target genes result in resistance to fluoroquinolones specifically, whereas alternations in efflux capability or permeability of organisms usually cause resistance to not only quinolones but also unrelated antibiotics, such as β -lactam drugs, tetracyclines, and chloramphenicol [20-23].

Alteration of Drug Targets

DNA gyrase and topoisomerase IV involved in DNA synthesis has been identified as the major targets of the fluoroquinolones because mutations in these genes

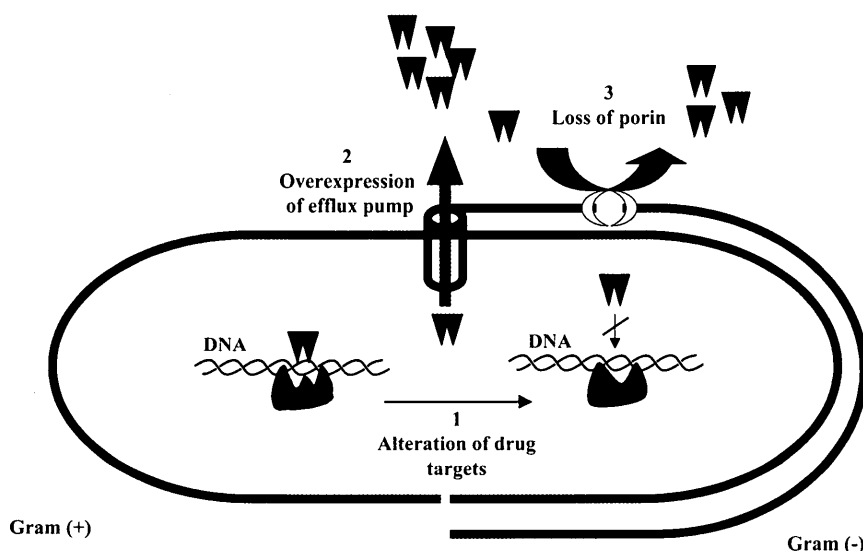


Fig. 2. Major molecular mechanisms of fluoroquinolone resistance: (1) alterations of target enzymes (DNA gyrase and/or topoisomerase IV) to prevent the drug binding; (2) overexpression of efflux pumps to decrease accumulation of drugs; and (3) loss of porins to reduce permeability of drugs in gram-negative bacteria. ▽ = fluoroquinolones; ⬤ = target enzymes, DNA gyrase, and/or topoisomerase IV; ⬤ = mutated target enzymes.

result in drug resistance [5]. In gram-negative bacteria, such as *E. coli*, GyrA subunit of gyrase protein is the primary target of fluoroquinolones, which is consistent with the original proposition before the discovery of topoisomerase IV in 1990 [8]. In some gram-positive bacteria, such as *Staphylococcus aureus*, ParC subunit of topoisomerase IV is the primary target for fluoroquinolones [24]. A mutation in topoisomerase IV, *grlA*

in *S. aureus*, also increases the minimum inhibitory concentration (MIC) of fluoroquinolones [25].

Resistance to fluoroquinolones in *E. coli* appears to be caused mainly by alterations in the *gyrA* gene of the DNA gyrase and in the *parC* gene of the topoisomerase IV (Table 2) [5,17,18]. Multiple mutations occur within a single chromosomal gene, resulting in mutants associated with variable MICs [17,18,26].

Table 1. Mechanisms of fluoroquinolone resistance in different organisms

| Species | Primary targets | Minor targets | Efflux components | | Permeability | References |
|-------------------------------|-----------------|------------------|--|--|----------------|-------------------------------|
| | | | Pump components | Regulatory gene or mutation | | |
| Gram-negative bacteria | | | | | | |
| <i>E. coli</i> | GyrA | GyrB, ParC, ParE | AcrAB-TolC EmrAB | <i>marR</i> , <i>marA</i> , <i>robA</i> , <i>soxS</i> <i>emrR</i> | OmpF, OmpC | [27,42,54-56,70-74] |
| <i>Salmonella</i> spp. | GyrA | GyrB | AcrAB-TolC | <i>marR</i> , <i>soxR</i> | | [26,75,76] |
| <i>Klebsiella</i> spp. | GyrA | ParC | | <i>ramA</i> | Ompk35, Ompk36 | [23,32,77,78] |
| <i>P. aeruginosa</i> | GyrA | GyrB | MexAB-OprM MexCD-OprJ MexEF-OprN | <i>mexR</i> <i>nfxB</i> <i>nfxC</i> | | [79-81] [82,83] [84,85] |
| <i>N. gonorrhoeae</i> | GyrA | ParC | | | | [86-88] |
| <i>Campylobacter</i> spp. | GyrA | | | | | [89,90] |
| <i>Helicobacter pylori</i> | GyrA | | | | | [91] |
| Gram-positive bacteria | | | | | | |
| <i>S. aureus</i> | GrlA | GyrA, GyrB | NorA | <i>flqB</i> | | [49,50,70] |
| <i>E. faecalis</i> | GyrA | | | | | [51] |
| <i>S. pneumoniae</i> | ParC | GyrA, GyrB | PmrA | | | [39,92-95] |
| Mycobacteria | | | | | | |
| <i>Mycobacteria</i> spp. | GyrA | GyrB | LfrA | | | [96] |

Table 2. Alterations in GyrA and ParC in *E. coli*

| Class | No. of isolates | MIC ($\mu\text{g/mL}$) | GyrA | | ParC | | References |
|-------|-----------------|--------------------------|-------|-----------------------|------------|------------------|---------------|
| | | | Ser83 | Asp87 | Ser80 | Glu84 | |
| 1 | 30 | 0.006-0.25 | | | | | [18,71,74,97] |
| 2 | 11 | 0.007-0.25 | | | | | [31,98] |
| 3 | 5 | 0.094-0.19 | | Asn, Gly, or Tyr | | | [18] |
| 4 | 1 | 0.032 | Ala | | | | [99] |
| 5 | 36 | 0.125-0.75 | Leu | | | | [18,74,99] |
| 6 | 1 | 2 | Trp | | | | [71] |
| 7 | 11 | 0.5-16 | Leu | | | | [71,97] |
| 8 | 8 | 0.25-4 | Leu | | | | [31,98] |
| 9 | 2 | 4-8 | Leu | | | Lys | [74,97] |
| 10 | 11 | 0.38-4 | Leu | | Arg or Ile | | [18,74,97] |
| 11 | 1 | 2 | Leu | | Ile | Val | [74] |
| 12 | 14 | 8-128 | Leu | Asn, Gly, or Tyr | | | [31,98] |
| 13 | 4 | 2-128 | Leu | Asn or Gly | | | [97] |
| 14 | 8 | 4-64 | Leu | Asn, Gly, or Tyr | | Lys | [18,71,74,97] |
| 15 | 4 | 8-256 | Leu | Asn or Gly | Arg | | [71,74,97] |
| 16 | 43 | 6-256 | Leu | Asn, Gly, His, or Tyr | Ile | | [18,71,74,97] |
| 17 | 9 | 32-128 | Leu | Asn or Tyr | Ile | Gly, Lys, or Val | [18,71,74,99] |

Different substitutions of one or several amino acids in the *gyrA* gene result in a wide range of ciprofloxacin MIC (from 0.32 to $\geq 256 \mu\text{g/mL}$). Most of the mutations in the *gyrA* gene are located in a small region of N-terminus of the GyrA protein (residues 67-106 in *E. coli*), called the quinolone resistance determining region (QRDR) [27], near the Tyr122, which binds to transiently cleaved DNA [9,12]. A similar QRDR has also been reported in *parC*. According to studies of mutant *E. coli* selected *in vitro*, mutations in *gyrA* result first in a substitution of Ser83 followed by Asp87 [28]. Results of surveys of mutant *E. coli* found *in vivo* appear consistent with the stepwise occurrence of mutations observed *in vitro*; nearly all single-site mutants have substitutions at Ser83 and most isolates with substitutions at Asp87 also have substitutions at Ser83 [5, 17,18].

Of fluoroquinolone-resistant clinical isolates, mutations in either *gyrA* or *parC* are more common than in either *gyrB* and *parE*. The most common substitution is Ser83 \rightarrow Leu83. In *Klebsiella pneumoniae*, discrepant amino acid, threonine [29,30] or serine [31,32], has been found at codon 83 of the GyrA protein in fluoroquinolone-susceptible isolates. Weigel *et al* [31] showed that the strain M5a1 with Thr83 amino acid position may be a strain of *Klebsiella oxytoca* and our data (unpublished) also showed that position 83 of GyrA in all quinolone susceptible isolates is serine. Both *Campylobacter jejuni* and *Pseudomonas aeruginosa* are 10-fold less susceptible to fluoroquinolones than wild-type *E. coli* because they contain a threonine in place of serine at position 83 of GyrA [33], underlining the

importance of Ser83 in fluoroquinolone resistance. However, not all mutations in *gyrA* confer resistance to all quinolones. Mutations Asp82Gly, Gly81Asp, Asp87Asn, Asp87Gly, or Asp87Tyr alone lead to a low-level resistance to fluoroquinolones [17,18,34].

According to the National Committee of Clinical Laboratory Standards (NCCLS) guidelines, MICs of ciprofloxacin $\geq 4 \mu\text{g/mL}$, $2 \mu\text{g/mL}$, and $\leq 1 \mu\text{g/mL}$ are considered as resistant, intermediate, and susceptible, respectively [35]. There are 2 populations of fluoroquinolone susceptible isolates: one is fully susceptible to fluoroquinolones and the other is reduced susceptible to fluoroquinolones [17,18,36]. Isolates with reduced susceptibility to fluoroquinolones had at least one mutation in *gyrA* at Ser83 position and were resistant to nalidixic acid, the first generation of quinolones. No strain possessed a *parC* mutation without the simultaneous presence of *gyrA* mutations in these studies (Table 2) [17,18]. These findings are consistent with fluoroquinolone resistance in *E. coli* arising in a multistep fashion with mutations in *gyrA* occurring as the first step [16]. In contrast, according to reports of some gram-positive bacteria, such as *S. aureus* and *Streptococcus pneumoniae*, mutants selected in a stepwise fashion with ciprofloxacin indicate that *parC* of topoisomerase IV can be the primary target of ciprofloxacin (Table 1) [24,37-39].

Overexpression of Efflux Pump

Reduced intracellular drug accumulation by pumping out the drug from the cell is one of the mechanisms that cause resistance. Even though mutations in drug

targets are the primary mechanism for fluoroquinolone resistance, increasing the activity of efflux pump also contributes to resistance, especially for high-level resistance. Efflux pumps involved in fluoroquinolone resistance also confer resistance to other non-structure-related antibiotics. The multiple antibiotic resistance (*mar*) locus has been reported to be responsible for fluoroquinolone resistance in *E. coli* [40]. A deletion or a point mutation in *marR*, a regulator gene of AcrAB efflux system, has been found in quinolone-resistant clinical *E. coli* isolates [41,42].

In *P. aeruginosa*, *nalB* [20], *nfxC* [43], and *nfxB* [44] mutants were resistant to quinolones due to overexpression of efflux pump systems MexA-MexB-OprM [45], MexE-MexF-OprN [46], and MexC-MexD-OprJ, respectively [21]. MexA-MexB-OprM efflux system was overexpressed in the *nalB* type mutants due to mutation in MexR, the repressor of MexA-MexB-OprM [47]. This type of mutation results in resistance to fluoroquinolones, carbenicillin, and tetracycline [20]. Both *nfxB* and *nfxC* type mutants were selected in mice infected with *P. aeruginosa* and treated with pefloxacin [48]. Mutation in the *nfxB* regulator gene resulted in overexpression of MexC-MexD-OprJ and caused resistance to fluoroquinolones, erythromycin, zwitterionic cepheems, and chloramphenicol [21,22]. These observations described above show that fluoroquinolones are substrates for these 3 efflux systems. Mutations in these efflux systems can decrease the susceptibility to fluoroquinolones from 2- to 8-fold dependent upon the type of fluoroquinolone [46].

Efflux pumps have also been identified in gram-positive bacteria. Mutation in *norA*, a multidrug efflux transporter, caused resistance not only to fluoroquinolones but also to ethidium bromide, acridine orange, tetracycline, and chloramphenicol in *S. aureus* [49,50]. An energy-dependent efflux was shown to pump out norfloxacin in both *Enterococcus faecalis* and *Enterococcus faecium* [51], and was associated with increased fluoroquinolone MICs in *S. pneumoniae* [52]. Recently, overexpression of *lfrA* efflux pump in mycobacteria was shown to confer fluoroquinolone resistance by decreasing drug accumulation *in vitro* [53].

Loss of Porins

Reduced intracellular drug accumulation by preventing the drug from entering the organism can contribute to resistance. Among gram-negative bacteria, porins form a channel for molecules traveling through cell membrane [54]. Decreasing the expression of outer membrane protein OmpF or OmpC affects uptake of fluoroquinolone in *E. coli* [54,55]. This is also the case

for *K. pneumoniae* and *E. coli*, in that mutation in *ompk35* or *ompk36* and homolog of *ompF* and *ompC*, respectively, results in decreased susceptibility to fluoroquinolones [23]. Recently, we have found that in addition to mutation in *gyrA*, mutation in the *ompk35* may be the primary mechanism of fluoroquinolone resistance in *K. pneumoniae* (unpublished data). However, the effect of porin loss on fluoroquinolone resistance is not universal. In *Salmonella*, isolates lacking OmpF porin showed no decrease in fluoroquinolone accumulation [56].

Fluoroquinolone Use

Fluoroquinolones are widely used in both human and veterinary medicine. Compounds such as ofloxacin and enrofloxacin administered to animals are either identical or similar to those of fluoroquinolone used in humans [57-59]. Antibiotics have been given to animals as growth promoters or for therapeutic treatments. Fluoroquinolone-resistant bacteria, such as *Salmonella* species, *Campylobacter* species, and *E. coli*, have been isolated from animals [60-62]. There is great concern that antibiotic-resistant organisms from animals can be transmitted to humans [59]. Even though there is no direct evidence to confirm this possibility, we should be aware that unrestricted use of antibiotics in both human and veterinary medicine contribute to selective pressure toward antibiotic resistance. To avoid the potential problem of antibiotic resistance, many countries prohibit antibiotics used in humans to be used in animals [63].

Clinical Impact of Fluoroquinolone-resistant Bacteria

Fluoroquinolone resistance in staphylococci is associated with susceptibility to methicillin. Less than 10% of methicillin-susceptible isolates are resistant to fluoroquinolones, whereas the resistance is notably more prevalent in the methicillin-resistant strains [64-66]. A prospective study of *K. pneumoniae* bacteremia found that 5.5% of isolates were resistant to ciprofloxacin (unpublished data). Extended-spectrum β -lactamase production was detected in 60% of these ciprofloxacin resistant isolates, compared with 16% of ciprofloxacin susceptible strains [67]. Recently, we have reported that resistance to other antibiotics was more common in ciprofloxacin-resistant isolates than in susceptible *E. coli* [17]. Patients infected with resistant isolates were more likely to have underlying cancer or received any antibiotic than patients infected with susceptible isolates [17].

Studies by researchers in our division have reported,

as part of a national surveillance program in Taiwan [36], that 11.3% (136/1203) and 21.7% (261/1203) of *E. coli* clinical isolates were resistant and reduced-susceptible to ciprofloxacin, respectively [17]. The interpretation of current NCCLS guidelines cannot distinguish reduced-susceptible *E. coli* from susceptible ones [68]. Clinical failures of infections by *Salmonella* species with reduced susceptibility to fluoroquinolones in patients treated with fluoroquinolones have been reported [69]. Thus, it is important to identify bacteria with reduced susceptibility to fluoroquinolone because they are prone to become resistant [17,18,59]. The NCCLS guidelines define that isolates with inhibitory zone diameters of ciprofloxacin of ≤ 15 , 16 to 20, and ≥ 21 mm are resistant, intermediate, and susceptible to ciprofloxacin, respectively. The breakpoint for the definition of fluoroquinolone resistance in *Neisseria gonorrhoeae* was adjusted recently due to reduced susceptibility [68]. *N. gonorrhoeae* with zone diameters of ciprofloxacin ≤ 27 , 28 to 40, and ≥ 41 mm are considered as resistant, intermediate, and susceptible to ciprofloxacin, respectively. We have recommended that current NCCLS breakpoints for fluoroquinolone resistance in *Enterobacteriaceae* be modified as follows: zone diameters of ciprofloxacin ≤ 15 , 16 to 29, and ≥ 30 mm be considered as resistant, reduced susceptible, and susceptible to ciprofloxacin, respectively [18]. In order to ensure that fluoroquinolones remain therapeutically effective, we have to be able to control the spread of reduced-susceptible isolates by identifying these isolates in hospital laboratories.

Acknowledgment

The authors would like to thank Ms. Tsai-Liang Lauderdale for suggestions on the manuscript.

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