REVIEW ARTICLE

Potential mechanisms of resistance to the modern fluorinated 4-quinolones

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Introduction

The 4-quinolones are unlike most agents of antimicrobial chemotherapy in that they are chemically synthesised compounds rather than naturally occurring products. The principal target of this class of drugs is the enzyme DNA gyrase (E.C. number 5.99.1.3) which is the only bacterial enzyme capable of introducing negative supercoils into DNA. DNA supercoiling plays an important role in bacterial metabolism because not only does it compact the chromosome but also it is involved in the regulation of gene transcription.

DNA gyrase is a tetrameric enzyme consisting of two A and two B subunits. The A subunit cuts both strands of DNA simultaneously at intervals four base-pairs apart and holds the strands apart but covalently bound to the enzyme. The B subunit, with ATP used for energy, then introduces a negative supercoil into the DNA and the A subunit then reseals the two strands. The 4-quinolones appear to bind to the A subunit and prevent the resealing of the DNA strands. The ability of DNA gyrase to break both DNA strands simultaneously means that not only is it able to supercoil DNA but also it can catenate and decatenate DNA and, hence, is involved in segregation of the daughter chromosomes after replication.

Although the 4-quinolones have been known for more than 25 years, their clinical use has been fairly limited until recently because of their narrow spectrum of activity and poor pharmacokinetics. The first therapeutically useful 4-quinolone synthesised was nalidixic acid the antimicrobial spectrum of which was limited to Enterobacteriaceae. Nalidixic acid has proved clinically useful in the treatment of urinary-tract and enteric infections. Over the past ten years there has been renewed interest in this class of antimicrobial agents spurred by the discovery that the addition of a piperazine side chain at C6 and fluoride at C7 to the common 4-oxo-1,4-dihydroquinoline skeleton caused a 1000-fold improvement in the antimicrobial activity of the compounds compared with the older drugs in this class. Thus, bacterial species hitherto refractory to the 4-quinolones were now within the compass of their therapy. Drugs such as pefloxacin, norfloxacin, ciprofloxacin, enoxacin and ofloxacin have been shown to be active against a wide range of clinical isolates at clinically achievable levels and are now available for use in various parts of the world. These new 4-quinolones appear to represent a significant advance in the control of bacterial infection. However, the ability of bacteria to develop resistance to the modern 4-quinolones will be a crucial factor in the ultimate success of this class of antimicrobial agents.

Plasmid-mediated 4-quinolone resistance

Bacterial resistance to an antimicrobial agent is usually mediated either via a plasmid-encoded factor or a chromosomal mutation. Plasmids are molecules of extrachromosomal DNA that can replicate independently of the chromosome and may be freely transferred between bacteria including those of different species. Plasmids can mediate antibacterial resistance by one of three mechanisms: (i) inactivation of the drug, e.g., the β-lactams; (ii) production of an additional target resistant to the antimicrobial agent, e.g., trimethoprim; (iii) active efflux of the drug from the cell, e.g., tetracycline.

It is usual for plasmids to carry a number of genes conferring resistance to many antibiotics. As a result of this, plasmids are usually the main carriers of resistance in many clinical bacteria and are a major cause of concern because they may lead to the rapid world-wide spread of resistance.

Plasmid-mediated resistance to the modern 4-
quino lunolones has not yet been identified. In the past few years two examples of plasmid-mediated nalidixic-acid resistance have been reported in Shigella dysenteriae strains isolated in Kashmir and in Bangladesh. However, neither report proved conclusively that nalidixic-acid resistance was transferable because they did not demonstrate that the resistance gene could be successively transferred from strain to strain. Indeed, whether the resistance to nalidixic acid was actually conferred by the plasmid has been questioned. Furthermore, neither of these plasmid-encoded nalidixic acid-resistance genes conferred resistance to the modern 4-quinolones, although one did appear to increase the frequency of resistance to ciprofloxacin. This fact has revealed the possibility that these plasmids may not actually carry a gene coding for nalidixic-acid resistance but rather are mutator plasmids increasing the frequency of chromosomal mutations to nalidixic-acid resistance. It has been suggested that this may explain the association of plasmids with nalidixic-acid resistance in Sh. dysenteriae strains isolated from Zaire.

From our previous knowledge it is difficult to envisage the mechanism by which plasmid-mediated 4-quinolone resistance could be expressed. The most likely mechanism would seem to be some sort of reduction of permeability. A drug-destruction mechanism appears unlikely as the 4-quinolones are synthetic agents and it seems unlikely, although possible, that an enzyme which destroys the drug exists. A 4-quinolone-resistant DNA gyrase encoded by a plasmid would also seem unlikely. The actual clinical relevance of this observation is difficult to envisage. Not only has plasmid-mediated resistance to the modern 4-quinolones not been identified but some bacteria containing R-plasmids have been shown to be hypersensitive to the 4-quinolones. Furthermore, the 4-quinolones appear to be plasmid-curing agents both in vitro and in vivo. However, despite the clinical reports of plasmid curing by the 4-quinolones, the actual clinical relevance of this plasmid-curing ability must be questioned as it seems unlikely that total elimination of plasmids could ever occur. The mechanism by which 4-quinolones eliminate plasmids may involve the selection of plasmid-free cells, as is the case with novobiocin, or, alternatively, plasmid replication may be more sensitive to the changes in supercoiling caused by the drugs than chromosomal replication.

Chromosomally-mediated 4-quinolone resistance

The only mechanism by which bacteria are able to develop resistance to these antimicrobial agents is through chromosomal mutations and it has been shown that chromosomally-mediated resistance to the 4-quinolones occurs by one of two mechanisms: either an alteration in the target enzyme, DNA gyrase; or a mutation causing a reduction in the permeability of the cell for the drug.

Alterations in DNA gyrase

In most species investigated so far, high-level resistance to all 4-quinolones appears to be conferred by mutations in the gyrA gene which codes for the A subunit of DNA gyrase thought to be the target of the 4-quinolones. gyrA mutations confer high-level cross-resistance to all 4-quinolones but do not seem to be associated with resistance to antibacterial agents which are unrelated to these drugs. Such mutations have been identified in Escherichia coli (cfxA, gyrA, nalA, nfxA), Pseudomonas aeruginosa (nalA, cfxA), Haemophilus influenzae, Citrobacter freundii and Serratia marcescens. An exception to this rule may be Bacteroides fragilis for which Kato et al. have reported that high-level resistance appears to be linked to a permeability change rather than to an alteration in the gyrA gene.

The gyrA genes of E. coli strain KL16 and of four spontaneous quinolone-resistant mutants of this strain have been mapped. The mutations which conferred quinolone resistance were all found to be within a small region near the N-terminus of the gyrA gene at nucleotides 248, 318, and 199. The amino-acid substitutions in the A subunit were serine to leucine or tryptophan, glutamine to histidine, and alanine to serine (at amino acids 83, 106 and 67, respectively). Recently, the gyrA gene of a clinical isolate of E. coli was sequenced and the amino-acid substitution was also at amino acid 83. It is interesting to note that all of these mutations are situated close to Tyr at amino acid 122 of the A subunit which has been shown to be the site, in DNA gyrase, that is covalently bound to DNA when the enzyme breaks the phosphodiestor bonds of DNA. However, until the three-dimensional structure of DNA gyrase has been determined the significance of this observation is unclear.

Mutations in the B subunit of DNA gyrase encoded by the gyrB gene may also affect bacterial susceptibility to the 4-quinolones. The

na131
mutation (also known as the nalC mutation) and the nal24 mutation, are point mutations in the gyrB gene that cause changes at amino acids 426 and 447 in the B subunit of DNA gyrase. Both mutations are in the region which Cozzarelli has postulated is involved in binding of the B subunit to the A subunit. The nal24 mutation causes the substitution of asparagine for aspartic acid whilst the nal131 mutation involves a change from lysine to glutamic acid. Both these amino-acid substitutions cause a change in the overall charge of the B subunit; the nal24 mutation decreases the negative charge of the protein, whereas the nal131 mutation has the opposite effect and increases the negative charge. This difference may explain the observation by Smith that the nal131 mutation confers resistance only to 4-quinolones that lack a C7 piperazine substituent, whilst rendering bacteria hypersensitive to 4-quinolones possessing a C7 piperazine. It has been suggested that this dichotomy results from the ability of the nal131 mutant strain to increase the negative charge on the B subunits of DNA gyrase, leading then to an increase in the attraction of the B subunit for the positively-charged piperazine group.

Most of the investigations of the effect of 4-quinolones on DNA-gyrase activity have been carried out with gram-negative bacteria and there have been comparatively few investigations done with gram-positive bacteria. It has been suggested from results obtained with Micrococcus luteus and Staphylococcus aureus that the DNA gyrase of gram-positive bacteria must inherently be much less susceptible to the modern 4-quinolones. However, Takahata and Nishino found that ciprofloxacin, norfloxacin and ofloxacin can inhibit the supercoiling activity of DNA gyrase from Staph. aureus and, therefore, it remains unclear how susceptible the DNA gyrase of gram-positive bacteria is to inhibition by the modern 4-quinolones.

4-quinolone resistance resulting from impermeability mechanisms

The ability of antimicrobial agents to penetrate bacteria is an important factor in their spectrum and activity. Therefore, reducing the ability of the 4-quinolones to penetrate into bacteria might decrease bacterial susceptibility to these drugs. The 4-quinolones penetrate bacteria by diffusion through the phospholipid bilayer and porins in the outer membrane of gram-negative bacteria. The hydrophobicity of the drug has an effect on its ability to diffuse across the phospholipid bilayer of the membrane. The more hydrophilic the drug, the less able it is to penetrate the bacterium through the phospholipid bilayer. The recent discovery of endogenous active-efflux of norfloxacin at the inner membrane of E. coli and P. aeruginosa suggests that the factors determining the entry of fluoroquinoles into bacteria may be more complicated than previously thought.

Mutations affecting 4-quinolone permeability have been identified in E. coli (nalB, nfxB, norB, cfxB), Salmonella, Pseudomonas (nalB, cfxB and nfxB, qr1, qr2), Klebsiella and Serratia. Unlike mutations in the gyrA or gyrB genes, which confer cross-resistance to other 4-quinolones only but not to unrelated antibacterial agents, mutations affecting 4-quinolone uptake may be associated with cross-resistance to unrelated antibacterial agents, such as tetracycline, chloramphenicol, the aminoglycosides and β-lactams. The impermeability mechanisms rendering bacteria resistant to the 4-quinolones have been extensively investigated in E. coli and in P. aeruginosa.

Impermeability in E. coli

Mutations in E. coli that confer resistance to the 4-quinolones by an impermeability mechanism appear to be associated with alterations in outer-membrane porin F (ompF), a protein coded by the ompF gene. Inactivation of the ompF gene results in resistance to the 4-quinolones. It is interesting to note that resistance resulting in alterations of the ompF porin has less effect on bacterial susceptibility to nalidixic acid than to the modern fluorinated drugs. This is probably because nalidixic acid, which is more hydrophobic than the modern drugs, is able to penetrate bacteria through the lipid bilayer as well as by the ompF porin.

Resistance to the 4-quinolones in E. coli may be mediated not only by an alteration in the ompF gene itself but also by mutations in regulatory genes controlling expression of ompF at a post-transcriptional level. Two mutations, nfxB mapping at 19 min and cfxB or norB mutations mapping at 34 min, have been shown to decrease ompF expression, although they do not map in the region of the ompF gene (which maps at 21 min). Again, cfxB appears to be an allele of the marA gene which confers resistance to tetracyclines and chloramphenicol as well as to the 4-quinolones. Hooper et al. suggest that the molecular mechanism results from the coding of a factor by marA which negatively regulates ompF expression. They suggest that cfxB is a
mutation which leads to an increase in the expression of this factor because cfxB is dominant over cfxB+.

Although nfxB is not an allele of marA, it is also associated with this gene because a functional marA gene is required for an nfxB mutant to remain viable. However, whether nfxB functions as a positive or negative regulator of ompF expression is not known.

Alterations of the outer-membrane proteins are not the only impermeability mechanism leading to 4-quinolone resistance. Changes in the lipopolysaccharides of the outer membrane are also associated with resistance. The norC mutation mapping at 8 min on the E. coli chromosome near the lac operon confers resistance to the 4-quinolones norfloxacin and ciprofloxacin as well as the ß-lactam cefoxitin. However the norC mutation also confers hypersusceptibility to hydrophobic drugs such as nalidixic acid, cloxacillin and novobiocin and to sodium dodecyl sulphate. Phage-susceptibility tests indicate that the norC mutation causes alterations in the lipopolysaccharide layer and ompF. However, it remains unclear how norC and the ompF protein interact. The hydrophobicity of the cell surface of the norC mutant was also increased. The norC mutation also appears to reduce levels of a second outer-membrane protein, ompC.

4-Quinolone resistance resulting from reduced drug uptake in E. coli may not be mediated solely by alterations in the outer-membrane proteins or phospholipid bilayer, as had been previously thought. Cohen et al. demonstrated a system in which norfloxacin may be actively pumped out of E. coli at the inner membrane. This suggests that 4-quinolone resistance resulting from decreased drug permeability is not caused solely by decreased diffusion of the drugs across the outer membrane but may be magnified by the active-efflux system at the inner membrane. Whether this active-efflux system is specific to norfloxacin or affects other 4-quinolones has not yet been elucidated.

However, other workers have found that energy inhibitors have little effect on the uptake of enoxacin and fleroxacin by E. coli suggesting that active efflux at the inner membrane may not involve all 4-quinolones. Hooper et al. have proposed that the discovery of this active-efflux system for norfloxacin suggests that another class of drug mutations causing 4-quinolone resistance may exist. This mechanism would be similar to tetracycline-resistance in which the drug-efflux system works with enhanced efficiency and, if the tetracycline-resistance analogy is continued, opens the possibility of a plasmid-mediated 4-quinolone-resistance mechanism resulting from a drug-efflux system.

Impermeability in P. aeruginosa

In P. aeruginosa 4-quinolone resistance has also been shown to be associated with alterations in outer-membrane proteins that may lead to cross-resistance to unrelated antibacterial agents. As with E. coli, some of these alterations in outer-membrane proteins confer less resistance to nalidixic acid than to the modern 4-quinolones. However, unlike E. coli, 4-quinolone resistance in P. aeruginosa does not appear to be associated with alterations in a specific outer-membrane protein but rather with alterations in a wide range of outer-membrane proteins.

Although resistance to the 4-quinolones in P. aeruginosa has been associated with the loss of outer-membrane protein F, alterations in a wide range of other outer-membrane proteins have also been described in 4-quinolone-resistant strains. Both the cfxB and nalB mutations caused alterations in the outer-membrane profile that were not related to ompF whilst Daikos et al. and Legakis et al. found that unstable low-level resistance to the 4-quinolones in P. aeruginosa was associated with the alteration or loss of a 31–32-Kda outer-membrane protein. However, unlike the ompF and cfxB mutations in P. aeruginosa, the loss or alteration of this 31–32-Kda protein did not confer cross-resistance to antibacterial agents which were unrelated to the 4-quinolones. Chamberland et al. found that low-level resistance to the 4-quinolones in P. aeruginosa (Qr1, Qr2) was associated with a reduction in the amount of outer-membrane protein G (ompG) and an antigenically related 40-Kda outer-membrane protein. The relationship and function of these two proteins is unclear but the alterations conferred resistance to some ß-lactams, chloramphenicol and tetracycline as well as to the 4-quinolones.

4-Quinolone resistance in P. aeruginosa is associated not only with the decreased expression of outer-membrane proteins but also with the appearance of a new protein. Hirai et al. demonstrated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis of outer-membrane proteins that norfloxacin resistance in the P. aeruginosa nfxB mutant strain was associated with the appearance of a 54-Kda protein. However, unlike the nalB and cfxB mutations, the nfxB mutation did not confer resistance to the ß-lactams or the aminoglycosides. On the contrary, it rendered the bacteria hypersusceptible to these drugs. Furthermore, the nfxB
membrane is not yet clear. However, recently, ciprofloxacin resistance in Staphylococcus aureus has been reported to result from reduced access of the drug to DNA gyrase. Whether this is related to reduced permeability through the cell membrane is not yet clear.

**Impermeability in other species**

Resistance to the 4-quinolones in Klebsiella, Enterobacter and Serratia spp. resulting from reduced drug uptake has also been associated with alterations in the outer-membrane proteins. However, impermeability mutants have not yet been identified in gram-positive bacteria. It has been suggested by Smith and Lewin that this may be because gram-positive bacteria lack an outer membrane and that that deficiency of an outer membrane, and its associated porins, may be advantageous to bacteria with respect to their susceptibility to these antibacterial agents. However, recently, ciprofloxacin resistance in Staphylococcus aureus has been reported to result from reduced access of the drug to DNA gyrase. Whether this is related to reduced permeability through the cell membrane is not yet clear.

**Frequency of resistance to the 4-quinolones**

When plasmid-mediated resistance does not exist (as is the case with the 4-quinolones), the frequency at which bacteria are able to develop resistance to an antibacterial agent is important in determining the efficacy of the drug. In-vitro experiments suggest that the frequency at which bacteria mutate to 4-quinolone resistance is much lower for the modern fluorinated 4-quinolones than for the older drugs in this class, such as nalidixic and oxolinic acids, Smith found that the mutation frequency in *E. coli* strain KL16 for resistance to the older 4-quinolones, such as nalidixic acid, was about $10^{-8}$ compared to less than $10^{-12}$ for the modern fluorinated drugs. Similar results with *E. coli* have also been obtained by other workers. It has been suggested that this may be because the modern drugs exert two distinct bactericidal mechanisms compared with only one for the older drugs.

An important factor in the ability of bacteria to develop 4-quinolone resistance is the concentration of 4-quinolone to which they are exposed. Cullmann et al. have shown that it is fairly easy to select for 4-quinolone resistance at concentrations near the minimum inhibitory concentration (MIC) (frequency about $10^{-6}$) but significantly more difficult at drug concentrations of $10 \times$ MIC. Hence, it would seem important to ensure adequate serum levels to prevent the emergence of resistance during therapy. In general, gram-positive bacteria have been able to mutate to 4-quinolone resistance at higher frequencies than gram-negative bacteria. An exception to this observation would appear to be *Enterococcus faecalis* as both Cullmann et al. and Chin and Neu were unable to detect any mutant strains of *Ent. faecalis* resistant to the 4-quinolones at concentrations of $10 \times$ MIC.

Although in-vitro experiments may give some idea of the frequency at which resistance to the 4-quinolones might be anticipated to develop, it is the selection of these mutants during clinical practice that really matters. In the table it can be seen that the frequency at which bacteria mutate to 4-quinolone resistance is much lower for the modern fluorinated 4-quinolones than for the older drugs in this class, such as nalidixic and oxolinic acids.
seen that the development of resistance to the modern fluorinated 4-quinolones during therapy has been shown to have occurred in a wide range of bacterial species. Somewhat surprising are reports of *E. coli* strains which have developed resistance to the modern fluorinated 4-quinolones, because it is very difficult to obtain resistant mutant strains of this species *in vitro*. However, the mechanisms of selection for resistant bacteria in clinical practice are difficult to mimic precisely in in-vitro experiments.

Such clinical reports indicate that it is possible for most bacterial species to develop resistance to the 4-quinolones during therapy. However, as most of these reports of resistance are isolated incidents, the interpretation of these reports to provide information on the frequency at which resistance is likely to develop clinically is difficult. The clinical trials conducted during the development of the 4-quinolones provide some information. The phase-II and phase-III trials of ciprofloxacin suggested that the frequency of resistance to the modern drugs might be low; thus, only in five out of 1993 infections treated with the drug were the causative organisms considered to have developed resistance to ciprofloxacin during treatment.

The monitoring of bacterial susceptibility to the 4-quinolones once introduced also serves to provide epidemiological information. Several large-scale studies have been carried out monitoring the susceptibility of bacterial pathogens to the 4-quinolones since they have become available and very little resistance to the modern 4-quinolones was observed; in general, resistance was <5%.

Kresken and Wiedemann monitored nalidixic-acid resistance in the Federal Republic of Germany, Austria and Switzerland from 1975 to 1986, with particular emphasis on the period between 1983 and 1986 when ofloxacin and norfloxacin became available in these countries. Despite a marked increase in the use of the 4-quinolones over that period, resistance in Enterobacteriaceae, *Staph. aureus* or *Ent. faecalis* did not increase. It should be noted that some variation in the percentage of nalidixic acid-resistant Enterobacteriaceae from different centres was observed. A particular problem was observed in Innsbruck where nalidixic-acid resistance was 12-1%, which appeared to result from 30-8% of *K. pneumoniae* being nalidixic-acid resistant. However, as a species *P. aeruginosa* was an exception, for between 1983 and 1986 the percentage of 4-quinolone-resistant strains increased from 3 to 10%. This is consistent with reports of the rapid development of ciprofloxacin resistance in *P. aeruginosa* in animal models and, clinically, elsewhere.

Another survey performed in the Federal Republic of Germany by Grimm, testing more than 100 000 bacterial strains isolated from hospitals and general practice between 1986 and 1987, found results similar to those of Kresken and Wiedemann for Enterobacteriaceae. Resistance to ofloxacin and ciprofloxacin was found in <1% of strains of different species in this family. In contrast to Kresken and Wiedemann, Grimm did not find an increase in resistance to the 4-quinolones in *P. aeruginosa*, although he did observe a significant increase in the incidence of 4-quinolone resistance in staphylococci particularly amongst *Staph. epidermidis*. This resistance in staphylococci was associated with multiple drug-resistance patterns and may be a cause for concern as animal models suggest that staphylococci may develop resistance to the 4-quinolones readily during therapy. Furthermore, a survey of methicillin-resistant *Staph. aureus* (MRSA) from various centres in the world found the incidence of ciprofloxacin resistance to be as high as 17%. An Israeli hospital has reported resistance to the 4-quinolones in 45 of 50 clinical isolates of MRSA, although none of the 20 MSSA tested during the same period were 4-quinolone-resistant. It should be noted, however, that none of the MRSA had actually developed resistance to the 4-quinolones during therapy.

In other bacterial species problems may also exist. The frequency of resistance to the 4-quinolones may be increasing in *B. fragilis*. A survey in Japan, in which more than 300 isolates of this species were examined, found that the frequency of 4-quinolone resistance was significantly higher in isolates collected in 1986–1987 than in isolates collected during 1983–1984.

At present, results from large-scale sensitivity surveys suggest that the incidence of 4-quinolone resistance in bacteria is still rare, although it seems to be occurring with increasing frequency in certain species such as *P. aeruginosa*, staphylococci (particularly multi-resistant organisms) and *B. fragilis*. It will be interesting to monitor the frequency of resistance to the 4-quinolones over the next few years as more 4-quinolones become available and the use of this class of antibacterial agents increases. It should also be noted that it has been suggested that 4-quinolone-resistant strains may be less pathogenic because of their reduced virulence. Further investigation of this phenomenon will be required before this is conclusively shown to be the case.
The 4-quinolones are broad-spectrum antibiotics for which plasmid-mediated resistance has not yet been identified. Bacteria are able to develop resistance to these drugs via chromosomal mutations which result in an altered target (DNA gyrase) or reduced uptake of the drug (associated with alterations in the outer membrane). Resistance to the 4-quinolones has been shown to occur clinically but its occurrence is still relatively infrequent. However, the incidence of resistance appears to be increasing in certain groups such as staphylococci and pseudomonads. It is to be hoped that, with appropriate use and dosing, the incidence of resistance to the modern fluorinated 4-quinolones will remain relatively low in the near future.

REFERENCES


93. Grimm H. Monitoring resistance to new quinolones and resistance patterns in the first two years following the introduction of these drugs. International Symposium on Ciprofloxacin, Dresden East Germany 1988.


Note added in proof

Ubukata et al\textsuperscript{102} have recently proposed that a recombinant plasmid, containing a 5.5-kb fragment of staphylococcal DNA believed to carry genes coding for a norfloxacin-resistant DNA gyrase, conferred resistance to both \textit{E. coli} and \textit{Staph. aureus}. This indicates that sensitivity may not always be dominant over resistance to the 4-quinolones in the gyrase genes. If this does prove to be the case, it seems possible that 4-quinolone resistance could be encoded by plasmids carrying 4-quinolone-resistant DNA gyrase genes. A possible explanation for this phenomenon is that the genes for the two subunits of DNA gyrase may be closely linked in staphylococci, unlike \textit{E. coli}, and that the recombinant plasmid carries genes for both subunits.