Eur J Clin Microbiol Infect Dis (2002) 21:647–659
DOI 10.1007/s10096-002-0788-z

REVIEW

F.-J. Schmitz · P.G. Higgins · S. Mayer · A.C. Fluit
A. Dalhoff

Activity of Quinolones Against Gram-Positive Cocci: Mechanisms of Drug Action and Bacterial Resistance

Published online: 7 September 2002

Abstract The quinolones are a potent class of antimicrobial agents that target two essential enzymes of bacterial cells: DNA gyrase and topoisomerase IV. Resistance is mediated chiefly through stepwise mutations in the genes that encode these enzymes, leading to alterations of the target site. These mutations occur in an area called the "quinolone resistance determining region". In gram-positive organisms, mutations occur more often in topoisomerase IV than in DNA gyrase. This target preference appears to depend upon two factors: the species of organism and the selecting drug. Resistance can be enhanced by a decrease in intracellular drug concentration, which is mediated through efflux pumps. The newer generation of fluoroquinolones and non-fluorinated quinolones exhibits enhanced activity against gram-positive organisms compared to the older members of this drug class, although development of resistance to these drugs has been demonstrated in vitro. This review gives a chronological perspective of the literature on the action of DNA gyrase and topoisomerase IV and the mechanisms of resistance to quinolones in staphylococci, streptococci and enterococci.

Introduction

A bacterium, for example *Escherichia coli*, is in the order of 2×1 μm in dimension and contains a chromosome of double-stranded DNA, which is 1300 μm in length [1]. In order to fit inside the bacterium, the DNA is negatively supercoiled and arranged around an RNA core. During transcription or DNA synthesis, the double-stranded DNA is unzipped to allow either messenger RNA or a new DNA strand to be synthesised. This unzipping of the DNA causes topological stress upstream of the RNA polymerase or replication fork and induces the formation of positive supercoils that need to be removed. To relieve this stress and to remove the positive supercoils, a topoisomerase known as DNA gyrase makes double-stranded breaks in the DNA and reduces the linking number by two. After DNA synthesis, the daughter chromosomes are unlinked by another topoisomerase, topoisomerase IV, in a process called decatenation. Both of these enzymes belong to the class of type II topoisomerases, use a double-strand-passage mode of action [2], are essential to bacteria and are the targets of the fluoroquinolones.

F.-J. Schmitz (✉) · P.G. Higgins · S. Mayer
Institute for Medical Microbiology and Virology,
Universitätsklinikum Düsseldorf, Universitätsstrasse 1,
Geb. 22.21, 40225 Düsseldorf, Germany
e-mail: schmitfj@uni-duesseldorf.de
Tel.: +49-2132-72040, Fax: +49-2132-72040

F.-J. Schmitz · A.C. Fluit
Eijkman-Winkler Institute for Medical Microbiology,
University Medical Center Utrecht, Utrecht, The Netherlands

A. Dalhoff
Institute for Medical Microbiology, Universitätsklinikum Kiel,
Kiel, Germany

DNA Gyrase

DNA gyrase is a heterotetramer composed of two A subunits and two B subunits, which are encoded, respectively, by the *gyr*A and *gyr*B genes. The GyrA subunits bind to DNA, and the GyrB subunits are ATPases. The most important physiological role of DNA gyrase is to maintain a level of DNA supercoiling that facilitates the movement of DNA through replication and transcription complexes. Furthermore, gyrase removes knots from DNA and helps to bend and fold DNA [3, 4]. The ability of gyrase to introduce negative supercoils results from the specific handedness of DNA binding to the enzyme. This is determined by the carboxyl terminal domain of the GyrA subunit, which is where the enzyme binds DNA and a segment of approximately 130 bp wraps around the tetramer [5]. Removal of the C-terminal region converts gyrase into an enzyme with strong decatenating activity, suggesting that wrapping favours intramolecular strand passage as opposed to intermolecular strand passage [5]. DNA is cleaved in each DNA

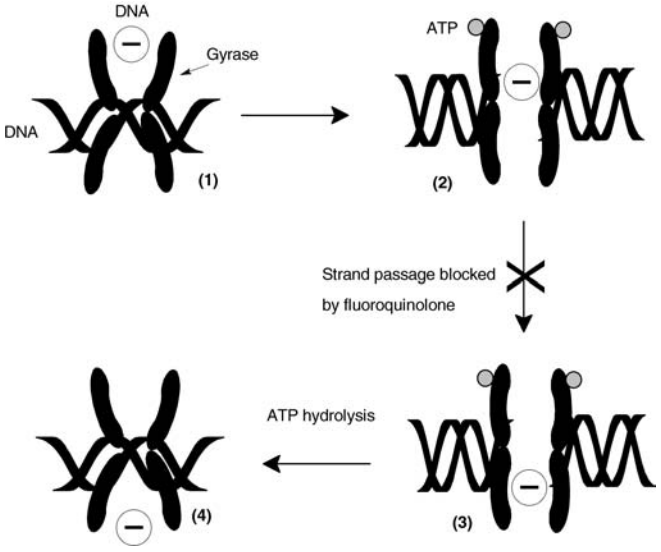

Fig. 1 Schematic diagram of strand passage. (*1*) DNA is in two planes, circle and dash coming out of the page, helix across the page. (*2*) ATP binding causes a conformational shift. DNA gyrase cleaves the double-stranded DNA and opens a gate. (*3*) The top strand passes through. Fluoroquinolones block strand passage. (*4*) The hydrolysis of ATP resets the enzyme for another round of strand passage. Adapted from [135]

strand, leaving a 4 bp staggered cut [6]. A covalent DNA-protein intermediate complex is formed between a DNA phosphate and a tyrosine residue (Tyr 122 in *Escherichia coli*) of GyrA. Bond energy is conserved and no energy co-factor is required for breakage or rejoining of DNA. The severed DNA ends come apart, and a gate opens to allow the passage of double-stranded DNA. Resealing of the DNA reduces the linking number by two, and the hydrolysis of ATP resets the enzyme for another round of supercoiling (Fig. 1) [7].

Gyrase is also able to remove negative supercoils from DNA in an ATP-independent manner [4, 8]. So, the amount of ATP is a key aspect in the supercoiling/relaxation relationship. Since the production of ATP is dependent on factors of extracellular environment, supercoiling is sensitive to factors such as salt concentration and oxygen tension [4, 9, 10, 11]. It has been shown that temperature [12, 13, 14] and pH [15] also influence supercoiling, but this might be ATP independent.

Temperature-sensitive alleles in either gene reduce chromosomal supercoiling and block initiation of replication at high temperature [16, 17]. Supercoiling is also reduced by inhibitors of gyrase and by reduction of function alleles that arise in response to a deficiency of topoisomerase I, the enzyme that prevents excess supercoiling from accumulating [4, 18, 19, 20]. These decreases in supercoiling increase gyrase expression, indicating that a homeostatic mechanism exists for the control of supercoiling [21].

Topoisomerase IV

Since 1990 it has been clear that topoisomerase IV is the enzyme that separates the bacterial daughter chromosomes (catenanes) after a round of replication [4, 22]. Like DNA gyrase, topoisomerase IV is a heterotetramer composed of two of each subunits ParC and ParE, encoded by the *par*C and *par*E genes (respectively, *grl*A and *grl*B in *Staphylococcus aureus*). The ParC and ParE subunits are homologous to GyrA and GyrB, respectively. In *Escherichia coli*, 36% of the ParC amino acids are identical to those found in GyrA, and 40% of ParE amino acids are identical to those found in GyrB [23]. In contrast to DNA gyrase, topoisomerase IV does not wrap DNA around itself [24]. It is, however, similar to eukaryotic topoisomerase II, in that bacterial topoisomerase IV might detect DNA crossovers and remove them, since topoisomerase IV is able to remove knots in DNA [22].

Topoisomerase IV also shows relaxing activity. It has been shown that overproduced topoisomerase IV is able to compensate for the loss of topoisomerase I, the main relaxing enzyme and counteractor of DNA gyrase [18]. Newer studies found topoisomerase IV to be a main agent together with topoisomerase I and DNA gyrase in generating the steady-state level of DNA-supercoiling. Moreover, it is also able to unknot DNA independently of supercoiling [25, 22].

The homology of structure and the functional similarities suggested that topoisomerase IV is also a target of quinolone antibiotics. This has been proved by studies demonstrating that quinolones can inhibit the DNA relaxation and decatenation activity of topoisomerase IV [26, 27, 28].

Mode of Action of the Fluoroquinolones on DNA Gyrase and Topoisomerase IV

The central event in the interaction between the fluoroquinolones and gyrase or topoisomerase IV is the formation of a quinolone-enzyme-DNA complex that contains broken DNA [8, 27]. The quinolones trap an intermediate during the catalysis reaction, leaving cut DNA attached to the topoisomerases (Fig. 1). In fact, it has been shown that fluoroquinolone binding is followed by a conformational change of the gyrase-DNA complex, which is responsible for the inhibition of the enzyme, and that cleavage of DNA is not necessary for the drug to bind to its target [29, 30]. The number of such complexes formed with gyrase correlates well with the extent to which overall DNA synthesis is inhibited. This is consistent with the idea that the complexes block movement of DNA through the replication machinery [31].

Biochemical work with gyrase has separated quinolone binding from subsequent trapping of cleaved complexes: a Tyr-122 substitution in gyrase blocks DNA cleavage but still allows binding of quinolone to the gyrase-DNA complex [6, 32, 33]. Moreover, limited prote-

olysis has revealed a quinolone-induced conformational change in the B subunit of mutant gyrase [30]. Since this conformational change differs from that generated by a Ca^{2+}-stimulated complex formation, two pathways for gyrase-dependent DNA cleavage probably exist [29]. In the quinolone-mediated pathway, ciprofloxacin affects the kinetics of ATP hydrolysis, giving rise to a characteristic rate that can be readily monitored [30]. Conversion to the quinolone-distinctive rate of ATP hydrolysis occurs quickly when quinolone is added to gyrase and DNA [34, 30]. DNA cleavage is a slower, subsequent process. Thus, quinolone binding to gyrase (assayed via the change-rate of ATP hydrolysis) and DNA cleavage are distinct steps.

Topoisomerase IV complexes might be similar to those formed with gyrase. Finer dissection of the quinolone-topoisomerase IV DNA reaction indicates that the quinolones stimulate DNA cleavage and inhibit religation [35]. Even in the absence of cleavage, they enhance both binding of topoisomerase IV to DNA and a distortion in the DNA of the complex [32]. Thus, for gyrase and topoisomerase IV, it is most likely that the quinolones interact with the enzymes and DNA before cleavage occurs; later, during enzyme catalysis, they interfere with the religation of DNA [34].

Inhibition of replication is not the reason for cell death, but cell death apparently occurs from the release of double-stranded DNA breaks from the drug-enzyme-DNA complex [4, 36]. Studies in which chromosomes are extracted from cells and examined with and without treatment with quinolones have shown that treatment with lower concentrations of quinolones leaves DNA able to form positive supercoils [28]. Therefore, the DNA breaks in the drug-enzyme-DNA complex must be constrained, so that DNA cannot rotate freely. Treatment of cells with supersaturating concentrations of quinolones with respect to complex formation and inhibition of DNA synthesis relaxes DNA [36]; negative supercoils are eliminated and positive supercoils cannot be introduced. This observation is consistent with the release of double-stranded DNA breaks from the drug-topoisomerase complexes. The quinolone concentration required to release the breaks is almost the same as that required to kill cells [4, 36].

Two models for lethal action are hypothesised. In one, lethal strand breaks are released from the collision of complexes with replication forks. In the other, lethal DNA breaks are released from complexes widely dispersed over the chromosome rather than being limited to complexes associated with replication forks. The idea of the fork collision model arose because topoisomerase IV-containing complexes can be dissociated by treatment with EDTA, but after collision with a replication fork they can no longer be separated by EDTA, and removal of these frozen complexes leads to cell death [31, 37]. Furthermore, upon challenge of *Escherichia coli* with nalidixic acid, DNA synthesis is inhibited but RNA synthesis is not, and filamentation of the cell occurs, signifying that cell division cannot take place [38]. However, this model is not representative of gyrase-mediated complexes. It fails to account for the little or no cell death that occurs during exposure of bacteria to quinolone concentrations sufficient to cause trapping of single-stranded DNA because inhibition of DNA synthesis correlates with the trapping of single-stranded rather than double-stranded DNA cleavage [28, 36]. Moreover, inhibition of intracellular DNA synthesis is reversible upon removal of quinolone [39], while cell death is not.

The dispersed model supports the hypothesis that cell death results from the release of double-stranded DNA breaks throughout the chromosome because lethal concentrations of oxolinic acid eliminate the ability of isolated nucleoids to maintain a supercoiled state [36]. This could not be true if frozen complexes were the only cause, because there are too few of these complexes on the chromosome and too many topological domains. Additional treatment of the cell with chloramphenicol hinders cell death and preserves supercoiling [36]. This observation suggests that quinolone treatment induces a protein responsible for the loss of supercoil constraint and subsequent cell death. The protein might act as a nuclease or a protease or as a chaperone that denatures the topoisomerase. However, the more potent fluoroquinolones, for example ciprofloxacin, are still able to kill cells in the presence of inhibitors of protein biosynthesis without a loss of supercoil constraint [34, 36, 40]. Thus, the lethal activity of the drug is dependent on the ability of the bacterial cells to maintain a supercoiled state. This property can be easily explained by the release of double-stranded DNA breaks that exist in widely dispersed complexes and by free rotation of the DNA. This second pathway of double-strand release might involve dissociation of the topoisomerase subunit. This idea is based on the observation that the quinolones stimulate a form of illegitimate recombination best explained by dissociation of the gyrase subunit [4, 36].

Another interesting fact is that, at lower concentrations, quinolones can act in a bacteriostatic way since they only block DNA replication, while at higher concentrations, they are bactericidal. In the fork collision model, bacteriostatic and bactericidal concentrations are equivalent [37], while in the dispersed model they are different.

Both models support the hypothesis that both gyrase- and topoisomerase IV-containing complexes can block cell replication and may cause cell death by means of processes such as SOS response and heat-shock response [4, 37, 41].

Mechanisms of Bacterial Resistance

There are three basic mechanisms of bacterial resistance against antibiotics: modification of the target site, inactivation of the drug, and altered intracellular concentration of the drug. With natural or semisynthetic drugs, mechanisms exist for all three strategies. The synthetic drugs, on the other hand, are believed to be indestructible to

enzymatic attack because an organism will never have encountered the drug "in the wild". The fluoroquinolones come under the latter category, and it is mutational events in the target site that lead to a decreased affinity of the target to the drug and altered intracellular drug concentration that account for resistance.

Modification of the Target Site

The most important mechanism of fluoroquinolone resistance is mediated through altered target enzymes. Importantly, resistance arises stepwise. In some species, first-step mutants map in *gyr*A and occasionally in *gyr*B, while in others they map in *par*C and less often in *par*E [4]. Recognition of this pattern led to the idea that, in some species, gyrase is the primary target, whereas in others, topoisomerase IV serves as the primary target [4, 42, 43, 44, 45, 46, 47, 48].

Alterations in DNA gyrase occur commonly in gram-negative bacteria and have been shown for many species to contribute to the resistance phenotype. Resistance mutations are found in GyrA more often than in GyrB, possibly reflecting the lower level of resistance conferred by GyrB mutations [4, 49]. The resistance mutations cluster in the "quinolone resistance-determining region" (QRDR) of GyrA, which is located between the amino acid positions 67 and 107 in *Escherichia coli* [50]. This is close to Tyr 122, which is transiently covalently bound to DNA phosphate groups during the enzyme-strand-passing reactions [50, 51, 52]. In the crystal structure of a 59 kDa fragment of GyrA, the positions of amino acids involved in the QRDR were localised to a positively charged surface along which DNA is thought to bind [51]. Thus, a common model shows that amino acids in the QRDR of GyrA alter the structure of the site of quinolone binding near the interface of the enzyme and DNA and that resistance is then caused by reduced drug affinity for the modified enzyme-DNA complex [53]. Resistant GyrB also shows a reduction in quinolone affinity [54]. This model might also be representative of the topoisomerase IV-enzyme-DNA interaction. The most important modifications in GyrA occur at positions 83 and 87 in *Escherichia coli* and at the homologous positions in other bacterial species [50]. In gram-negative species, GyrA is the primary target of 4-quinolones, as the first mutations conferring resistance occur in *gyr*A and single *par*C mutations do not change the MIC of quinolones for *Escherichia coli* [42]. In contrast to this, in gram-positive bacteria, topoisomerase IV can be the primary target, with gyrase serving as a secondary target [45, 46, 47, 48, 55].

Staphylococcus aureus *and Coagulase-Negative Staphylococci*

In 1990 Shreedhan et al. [43] identified resistance mutations in the *gyr*A gene of *Staphylococcus aureus* that lead to the amino acid substitutions Ser84-Leu and Ser85-Pro (for a list of common mutations, see Table 1). These mutations are analogous to the Ser83-Leu and Ala84-Pro mutations in *Escherichia coli*. In 1994 Ito et al. [49] characterised the mutations in *gyr*A and *gyr*B, which confer resistance in *Staphylococcus aureus*. They found the Ser84-Leu and Ser85-Pro substitutions as well as a Ser84-Ala and a Glu88-Lys change in GyrA. In GyrB they were able to detect the change of Asp437-Asn and Arg458-Gln. In 1994 Ferrero et al. [56] cloned topoisomerase IV and demonstrated it to be a primary target of the quinolones. They detected resistance mutations in *grl*A that lead to a Ser80-Phe or Ser80-Tyr change in the GrlA subunit of topoisomerase IV. This was confirmed by Yamagishi et al. [57], who found two GrlA alterations responsible for resistance: Ser80-Phe and Glu84-Lys. In 1995 Ferrero et al. [56] concluded that, based on stepwise selection with ciprofloxacin in which first-step mutants showed the GrlA alterations Ser80-Phe/Tyr and Glu84-Lys, topoisomerase IV is the primary target of fluoroquinolones in *Staphylococcus aureus*.

Second-step mutants exhibited a reduced accumulation of norfloxacin or a second mutation in GyrA, namely Ser84-Leu or Glu88-Lys [48]. These results were confirmed by Ng et al. [45], who found in first-step ciprofloxacin- and ofloxacin-selected mutants only point mutations in *grl*A (corresponding to Ser80 and Ala116-Pro/Glu) but no mutation in *gyr*A. In genetic outcrosses, a GyrA mutation expressed resistance only in GrlA mutants, which confirmed topoisomerase IV as the primary target of fluoroquinolones. After Tanaka et al. [58] showed in 1995 that quinolone-resistant clinical isolates had alterations in GyrA or enhanced efflux, they examined the GyrA and GrlA of clinical isolates and detected the same resistance mutations in *gyr*A and *grl*A as in vitro selected mutants [59]. Following studies with large numbers of isolates, Schmitz et al. [60, 61], Takahashi et al. [62] and Wang et al. [63] showed the Ser80-Phe/Tyr alteration in GrlA and the Ser84-Leu change in GyrA to be the principal ones, with GrlA single mutants conferring low-level resistance and a combined GyrA-GrlA double mutation conferring high-level resistance (Table 1).

Fournier and Hooper [64, 65] characterised mutations that differed from those previously described in that they exhibit cross-resistance against quinolones and coumarins. The coumarin antibiotics act via competitive inhibition of ATP hydrolysis by the B subunit of DNA gyrase. A mutation in GrlB at position 470 (Asn→Asp) confers resistance against quinolones and increases susceptibility to coumarins. In which way this mutation influences the interaction between enzyme, DNA and quinolone is not yet clear [65]. This interesting phenotype of quinolone resistance and coumarin hypersuceptibility can also be exhibited by mutant strains carrying the GrlA substitution Ala116-Glu/Pro [64]. This *grl*A mutation, in contrast to those localised in the common QRDR, is closer to the active site of enzyme breakage of DNA, suggesting possible effects on enzyme function.

Table 1 Common mutations in GyrA and ParC/GrlA and the corresponding MICs of ciprofloxacin for clinical isolates of *Staphylococcus aureus*, *Enterococcus faecium*, *Enterococcus faecalis* and *Streptococcus pneumoniae*

Organism	Amino acid change		Ciprofloxacin MIC (mg/l)	Reference
	GyrA	ParC/GrlA		
Staphylococcus aureus	–	–	0.25	[56]
	–	Ser80→Phe/Tyr	2–16	[60, 63]
	Ser84→Leu	nt	16	[43]
	Glu88→Lys	Ser80→Phe	8–32	[60]
	Ser84→Leu	Ser80→Tyr	16	[60]
	Ser84→Leu	Ser80→Phe/Tyr	12.5–800	[63]
	Ser84→Leu	Glu84→Lys	100–800	[63]
	Ser84→Leu	Ser80→Phe/Tyr + Glu84Lys	100–>800	[63]
	Glu88→Lys	Ser80→Phe + Glu84Val	32–256	[60]
Enterococcus faecium	–	–	1–8	[111, 112]
	–	Ser80→Ile	64–256	[112]
	Glu87→Lys	Ser80→Ile	128–256	[111]
	Ser83→Ile	Ser80→Arg	64	[111]
	Ser83→Leu	Glu84→Thr	16–32	[112]
	Glu87→Lys	Ser80→Ile	32–>256	[111]
	Glu87→Gly	Glu84→Lys	256–>512	[112]
Enterococcus faecalis	–	Ser80→Arg	3.13	[136]
	Ser83→Arg	Ser80→Ile	25	[136]
	Glu87→Gly	Ser80→Ile	50	[136]
	Ser83→Ile	Ser80→Ile	100	[136]
Streptococcus pneumoniae	–	–	1	[100]
	Ser81→Phe	–	1	[97, 100]
	Ser81→Tyr	–	1	[100]
	–	Ser79→Phe	8	[44, 100]
	–	Ser79→Tyr	8	[100]
	Ser81→Phe	Asp83→Asn + Lys137→Asn	4	[91]
	Ser81→Phe	Ser79→Phe	8–>64	[91]
	Ser81→Phe	Ser79→Phe + Lys137→Asn	8–16	[46]
	Ser81→Tyr	Ser79→Phe	32–128	[46]

–, no mutation; nt, not tested

To examine the target preference of the quinolones, decatenation and supercoiling assays were performed by Tanaka et al. [66] with levofloxacin, DR-3354, DU-6959, DV 7751a, ciprofloxacin, tosufloxacin and sparfloxacin and by Blanche et al. [67] with ciprofloxacin and sparfloxacin. These studies showed decatenation (mediated through topoisomerase IV) to be more susceptible to fluoroquinolone inhibition in *Staphylococcus aureus*. In contrast, *Escherichia coli* DNA gyrase supercoiling is more susceptible. However, mutated *Staphylococcus aureus* topoisomerase IV is less sensitive to fluoroquinolones. In 1999 Gootz et al. [68] confirmed – using fluoroquinolone selection, sequencing, decatenation, supercoiling and cleavage experiments – that topoisomerase IV is the primary target for the fluoroquinolones trovafloxacin, levofloxacin, sparfloxacin, pefloxacin and ciprofloxacin. These results emphasised that topoisomerase IV is the primary target of fluoroquinolones in *Staphylococcus aureus*.

Contrary to the results reported by Gootz et al. [68], Ruiz et al. [69] described the selection of mutants with sparfloxacin in which the primary mutation was located in the *gyr*A gene. Furthermore, Takei et al. [70] examined the target preference of 15 quinolones against *Staphylococcus aureus* GyrA and GrlA mutant strains (Ser84-Leu and Ser80-Phe, respectively) and noticed a significant correlation between the MIC ratios of the GyrA strain to the GrlA strain and the IC50 ratios. This led them to postulate that the target preference in the wild-type strain can be anticipated by the MIC ratios. On the basis of these ratios, the quinolones were classified into three categories: type I quinolones (norfloxacin, enoxacin, fleroxacin, ciprofloxacin, lomefloxacin, trovafloxacin, grepafloxacin, ofloxacin and levofloxacin) had MIC ratios of <1, type II quinolones (sparfloxacin and nadifloxacin) had MIC ratios >1, and type III quinolones (gatifloxacin, pazufloxacin, moxifloxacin and clinafloxacin) had MIC ratios of 1. Type I and type II quinolones seem to prefer topoisomerase IV and gyrase, respectively, while type III quinolones seem to target both enzymes at nearly the same level in bacterial cells.

In 2000 Ince and Hooper [71] selected mutants with premafloxacin and found a new substitution inside the QRDR in GrlA (Ala76-Thr) and three new mutations outside the GrlA QRDR: Arg43-Cys, Asp69-Tyr and Pro157-Leu. More recently, Ince and Hooper [72] underlined the importance of the 8-methoxy substituent by comparing gatifloxacin, its desmethoxy derivative and ciprofloxacin. Gatifloxacin was found to be most active against mutants of *Staphylococcus aureus*, and a novel

substitution outside the QRDR of GrlA was detected: Lys23-Asn. Two novel mutations in *grl*B were also found: Pro25-His and Pro451-Gln. Allelic exchange experiments confirmed the role of the novel mutations in resistance, suggesting that the QRDR in GrlA should be expanded to include these mutations. Recently, interest has focused on nonfluorinated quinolones (NFQs).

Roychoudhury et al. [73] examined the effects of three NFQs on cell growth and compared these effects with those of the fluoroquinolones ciprofloxacin, trovafloxacin, gatifloxacin and clinafloxacin. They found the NFQs, clinafloxacin and gatifloxacin to have a greater ability to exploit both DNA gyrase and topoisomerase IV. Furthermore, the NFQs and clinafloxacin were less susceptible than the other fluoroquinolones to existing mechanisms of quinolone resistance in *Staphylococcus aureus*, as shown by MIC and kill kinetics. Discotto et al. [74] suggested, on the basis of the results of in vitro selection with BMS-284756, that gyrase is the primary target of NFQs in *Staphylococcus aureus*. However, Roychoudhury et al. [75] recently isolated mutants by stepwise selection with fluoroquinolones and three NFQs (PGE 9262932, PGE 4175997 and PGE 9509924) and found the first-step mutation to occur in *grl*A (Ser80-Phe). Examination of three third-step mutants revealed some new mutations. Two of these mutants with high-level NFQ resistance showed, in addition to the Ser84-Leu alteration in GyrA and the Glu84-Lys alteration in GrlA, the alteration His103-Tyr in GrlA; one also had a mutation in *grl* B (Glu472-Val). One of the third-step mutants had a Ser84-Leu alteration in GyrA, a Glu477-Val change in GyrB and a change from Ser52-Arg in GrlA [75].

Shreedhan et al. [76] showed in 1991 that the GyrA Ser 84 alteration is common in coagulase-negative and coagulase-positive staphylococci. Dubin et al. [77] found that the mutations seen in coagulase-negative staphylococci such as *Staphylococcus epidermidis*, *Staphylococcus hominis* and *Staphylococcus haemolyticus* at positions 80 in GrlA and 84 in GyrA are similar or identical to those seen in *Staphylococcus aureus*: Ser80-Phe/Tyr in GrlA and Ser84-Leu in GyrA, depending on whether the wild-type codons are TCT or TCA. These results were confirmed by Linde et al. [78]. In *Staphylococcus hominis* a Ser84-Ala substitution in GyrA is uncommon, and the GrlA Asp84-Gly substitution in *Staphylococcus haemolyticus* resembles Glu84-Gly. In *Staphylococcus epidermidis* the amino acid equivalent of Glu84 in ParC is aspartate, and Li et al. [79] identified changes in this residue from Asp to Asn, Ala or Tyr in the most resistant isolates in conjunction with a Ser84 alteration in both GyrA and ParC.

Streptococcus pneumoniae, Streptococcus pyogenes *and Viridans Group Streptococci*

The examination of the resistance mutations in streptococci began in 1996, when Pan and Fisher [55] selected *Streptococcus pneumoniae* mutants stepwise with ciprofloxacin and found an alteration in ParC (Ser79-Tyr) that conferred low-level resistance. High-level resistance was acquired though mutations in *par*C and *gyr*A [47]. In further studies, stepwise selection with ciprofloxacin showed the acquisition of additional mutations in *par*C before *gyr*A and *gyr*B. The alterations identified were Ser79-Tyr/Phe and Ala84-Thr in ParC, Ser81-Tyr and Asp85-Lys in GyrA and Asp435-Asn in GyrB [44, 80]. Munoz and de la Campa [46] sequenced the *par*C gene from clinical isolates and identified the same resistance mutations formerly found in mutants selected in vitro (Table 1). These data emphasised that topoisomerase IV seemed to be the primary target of fluoroquinolones in *Streptococcus pneumoniae*. Isolates selected in vitro and in vivo were also sequenced by Tankovic et al. [81], who identified, in addition to the already known substitutions, an Asp83-His alteration in ParC. Later they identified a mutation in *par* E, which changed Asp435-Asn, in a resistant isolate that had no mutation in the other target genes [82]. However, drug efflux was not investigated in this isolate, and the role of the mutation is speculative.

In 1997 Pan and Fisher [83] overturned the claim that topoisomerase IV is the primary target of *Streptococcus pneumoniae* when they found a primary mutation in *gyr*A upon selection with sparfloxacin. This discovery suggested that the target preference is dependant on the selecting fluoroquinolone, as previously discussed with *Staphylococcus aureus*. Later on they identified gyrase and topoisomerase IV to be dual targets of clinafloxacin [84]. In GyrB and ParE they found novel alterations at codons 474 (Glu→Lys) and 454 (Pro→Ser), respectively. A single GyrB Glu474-Lys mutant, which had no other mutations in GyrA, ParC or ParE, conferred low-level resistance to sparfloxacin and clinafloxacin but not to ciprofloxacin. Fukuda and Hiramatsu [85] also selected GyrA first-step mutants with sparfloxacin and gatifloxacin. This contrasted with their selection of a *par*C mutation with trovafloxacin, levofloxacin, norfloxacin and ciprofloxacin. The target preference of trovafloxacin can be questioned on the basis of the results of Davies et al. [86], who found trovafloxacin *gyr*A mutants without any alteration in *par*C. Davies et al. [86] also described the incidence of quinolone-resistant pneumococci as presently being very low amongst a range of penicillin-susceptible and -resistant pneumococci. Varon et al. [87] considered the MICs of fluoroquinolones and the mutations in the target enzymes and assumed that ParC is the primary target of trovafloxacin and pefloxacin, while both enzymes may be initial targets of ciprofloxacin, sparfloxacin, moxifloxacin, Bay3118 and grepafloxacin.

In 1998 Taba and Kusano [88] examined sparfloxacin and ciprofloxacin resistance in clinical isolates and found the Ser81-Phe and Glu85-Lys substitutions in GyrA and the Ser79-Phe/Tyr and Lys137-Asn substitutions in ParC. Several more studies have confirmed these results [89, 90, 91]. In 1999 Stewart et al. [92] tested the relationship between mutations in *par*C and *gyr*A of clinical isolates and resistance to ciprofloxacin and grepafloxacin. Interestingly, grepafloxacin resistance

seems to depend only on a *gyr*A mutation, and no relationship was detected between the MICs of grepafloxacin and the mutation of the QRDR of *par*C. Recent work has shown that grepafloxacin selects for *gyr*A mutations before *par*C [93]. Janoir et al. [94] described a new mutation in *par*E (His102Tyr) outside the QRDR in the putative ATP-binding site of topoisomerase IV. As selection experiments had revealed, the question of the primary target in *Streptococcus pneumoniae* seems to be dependent on the quinolone. Thus, it was interesting to examine whether the target preference can also be seen in the inhibition of both enzymes.

Using purified enzymes, Pan and Fisher [95] examined the inhibitory effects of ciprofloxacin, sparfloxacin and clinafloxacin and found all three quinolones were more active in inhibiting purified topoisomerase IV than gyrase. Interestingly, the agents were at least 25-fold more effective in stabilising a cleavable complex with topoisomerase IV than with gyrase in spite of the previously discussed target preferences [83, 85]. The equipotency of ciprofloxacin and sparfloxacin in these assays are in stark contrast to their MIC values, as sparfloxacin is fourfold more potent than ciprofloxacin (MICs of 0.64 and 2.7 mg/l, respectively) [95]. These results were confirmed by Fernandez-Moreira et al. [96].

Pan and Fisher [95] explained this contrast in terms of a model for bacterial killing by quinolones in which cellular factors can modulate the effects of target affinity to determine the cytotoxic pathway. These cellular factors, which include DNA template, Mg^{2+} concentration, salt, polyamine and ATP, all found within a bacterium, may be difficult to reproduce in vitro.

More recently, Pan et al. [97] compared DNA gyrase and topoisomerase IV with altered GyrA (Ser81-Phe) and ParC subunits (Ser79-Phe) by enzyme inhibition and cleavage assays against ciprofloxacin and sparfloxacin. Both these agents inhibited purified mutant enzymes 8- to 16-fold less than wild-type enzymes. However, the MICs of these agents for the isolates carrying the mutations did not follow this pattern. The *gyr*A mutant was resistant to sparfloxacin but sensitive to ciprofloxacin, and the *par*C mutant was resistant to ciprofloxacin but sensitive to sparfloxacin. This suggests that fluoroquinolone-mediated killing involves other, as yet unknown, cellular factors.

Regarding gemifloxacin, Fisher et al. [98] found this substance to target both gyrase and topoisomerase IV because single mutations in either enzyme resulted in an (marginally) increased MIC. However, selected first-step mutants had a mutation in *gyr*A, whereas the second-step mutants exhibited an additional mutation in *par*C. Thus, gyrase seems to be the preferential target in vitro. Fisher et al. [98] could also demonstrate that gemifloxacin was more effective than ciprofloxacin in stabilising a cleavable complex with either gyrase or topoisomerase IV in vitro, which might be a reason for the greater potency of gemifloxacin. Nagai et al. [99] compared gemifloxacin with trovafloxacin, ciprofloxacin, gatifloxacin and moxifloxacin and showed that gemifloxacin selects less resistant mutants than the other fluoroquinolones. Primary mutations occurred in *gyr*A, *par*C or *par*E, so both enzymes were also thought to be the target of gemifloxacin.

Alovero et al. [100] found that the C-7 substituent determines not only the potency but also the target preference of fluoroquinolones. They examined the antipneumococcal mechanisms of a series of novel fluoroquinolones that are identical to ciprofloxacin except for the addition of a benzenesulfonylamido group to the C-7 piperazinyl ring. A substituent bearing a 4-(4-aminophenylsulfonyl)-1-piperazinyl group at C-7 showed enhanced activity against a pneumococcal strain and, in contrast to ciprofloxacin, a mutation in *par*C (Ser79-Phe/Tyr) could not confer resistance against the new substance, while a mutation in *gyr*A (Ser81-Phe/Tyr) made the mutant strain four- to eight-fold more resistant against it. Moreover, the new substance selected a *gyr*A mutant and showed enhanced activity in inhibiting supercoiling.

Regarding the C-8-methoxy substituent, Fukuda et al. [101] demonstrated its importance by comparing gatifloxacin and AM-1147 (also a C-8-methoxy quinolone) and their respective 8-H counterparts. They showed the 8-methoxy derivatives to select mutants at a lower frequency than their 8-H counterparts and to prefer DNA gyrase. Furthermore, the 8-methoxy derivatives showed a higher activity against target-altered mutant strains than the wild-type strain.

Focusing on the NFQs, Roychoudhury et al. [73] described the NFQs to be more active against *Streptococcus pneumoniae* than ciprofloxacin, trovafloxacin and gatifloxacin, and comparable to clinafloxacin. Hartman-Neumann et al. [102] showed the NFQ BMS 294756 to select mutants with the known changes in GyrA (Ser81 and Glu85) and ParC (Ser79 and Asp83).

The transfer of resistance determinants between *Streptococcus pneumoniae* and viridans streptococci has been demonstrated in vitro [103]. The frequency of transfer correlated with the homology of the *Streptococcus pneumoniae* QRDR and that of the donors' strains, with *Streptococcus oralis* having the highest frequency of transfer. A ParC resistance mutation could also be transferred from *Streptococcus pneumoniae* into *Streptococcus mitis* or *Streptococcus oralis*. Moreover, high-level resistant *Streptococcus pneumoniae* transformants resulted by means of the simultaneous transfer of mutated *par*C and *gyr*A genes from *Streptococcus mitis* in one step at low frequencies. Because of these results, the authors anticipate the dissemination of a resistant species in the clinical setting. In 2000 Ferrandiz et al. [104] reported the horizontal transfer of *par*C and *gyr*A in fluoroquinolone-resistant clinical isolates of *Streptococcus pneumoniae*. Bast et al. [105] supported the occurrence of interspecies recombination but found the contribution of interspecies recombination of type II topoisomerase genes among clinical isolates and the emergence of quinolone resistance to be minimal.

In 1998 Gonzalez et al. [106] examined clinical isolates of viridans group streptococci. They found the

Ser79-Phe/Ile alteration in ParC, and in GyrA they identified the Ser81-Phe/Tyr substitution. As novel substitutions, they found a mutation in ParE at codon 424 (Pro→Gly) in *Streptococcus mitis*. A recent study by Kaneko et al. [107] on oral streptococci did not reveal any new mutations, although ofloxacin selected less resistant mutants than DU-6859a and selected different amino acid substitutions in GyrA and ParC. Recently, Guerin et al. [108] identified an additional *par*E mutation (changing Glu474-Lys) in *Streptococcus mitis*.

In November 2000 Yan et al. [109] identified the first quinolone resistance mutations in *Streptococcus pyogenes*. Not surprisingly, they were located at the hot spots Ser81 in GyrA and Ser79 in ParC and resulted in a change to Phe and Tyr, respectively. They also identified a point mutation that resulted in a change from methionine to leucine at position 99, but the contribution of this mutation to resistance is not clear.

Enterococcus faecium *and* Enterococcus faecalis

Less work has been performed with the enterococci. In 1996 Tankovic et al. [110] identified among clinical isolates of *Enterococcus faecalis* a GyrA substitution at Ser83 to Arg or Ile and at codon 87 from Glu to Gly (Table 1). Li et al. [79] further identified in 31 clinical isolates ParC alterations at position 80 (Ser-Arg/Ile) and 84 (Glu→Ala) and an additional change in GyrA at codon 87 (Glu→Gly) (Table 1). In *Enterococcus faecium*, El Amin et al. [111] detected two resistance mutations in GyrA located at positions 83 and 87 (Ser→Tyr, Arg or Ile; Glu→Lys, respectively) and in ParC a Ser80-Ile change. Brisse et al. [112] examined 73 quinolone-resistant clinical strains and identified additional mutations at codon 87 from Glu to Gly and at codon 83 from Ser to Leu in GyrA. Furthermore, they showed a change from Glu84 to Lys/Thr. Six of the isolates had amino acid changes in ParC alone, without an additional change in GyrA. This may indicate that topoisomerase IV is the primary target of fluoroquinolones within these organisms.

The similarity of mutations in GyrA and ParC found in clinical isolates of *Staphylococcus aureus*, *Enterococcus faecalis*, *Enterococcus faecium* and *Streptococcus pneumoniae* is noteworthy (Table 1).

Efflux Pumps and Their Role in Fluoroquinolone Resistance

The second resistance mechanism in gram-positive bacteria is mediated through active drug efflux by the overexpression of certain efflux pumps. These efflux pumps are multidrug transporters that are able to transport, for example, fluoroquinolones, ethidium bromide, chloramphenicol, tetraphenylphosphonium and rhodamine 6G. Resistance is due to active transport driven by the proton motive force; therefore, energy inhibitors like carbonyl cyanide m-chlorophenylhydrazone abolish the transport process. In 1989 Ubukata et al. [113] cloned and expressed the *nor*A gene of *Staphylococcus aureus*. One year later Ohshita et al. [114] identified a point mutation in the *nor*A gene at position 209, changing adenosine to cytosine, which was thought to be responsible for quinolone resistance. Neyfakh et al. [115] determined that the efflux pump NorA of *Staphylococcus aureus* belongs to the MFS, the major facilitator superfamily, and is very similar to BmrA of *Bacillus subtilis*, consisting of 12 predicted membrane-spanning domains.

Strains of *Staphylococcus aureus* that become resistant to fluoroquinolones because of increased efflux can be divided into two classes. The first exhibits inducible overexpression of NorA. Kaatz and Seo [116] demonstrated an inducible NorA that did not have a mutation in either *nor*A or the flanking DNA, leading to speculation of the involvement of other loci, most probably a regulator. The second one exhibits constitutive overexpression of NorA, which might be related to mutations 89 bp upstream from the putative ATG start codon (the *flq*B locus). In 1993 Kaatz et al. [117] examined the transport process and demonstrated by Southern and Northern blotting that increased transcription and not gene amplification is responsible for fluoroquinolone resistance. In 1994 Ng et al. [118] showed the *flq*B resistance locus of *nor*A was associated with increased expression of *nor*A. This mutation increases the half-life of *nor*A mRNA transcripts, and Fournier et al. [119] have hypothesised that an additional stem-loop in the mutated transcripts protect against RNAse III. In 1997 Yamada et al. [120] showed that NorA plays a role even in quinolone-susceptible strains, since *nor*A disruption led to MICs eightfold lower than those for the parent strain.

Previously, only hydrophilicity of the fluoroquinolones was thought to be an important factor for NorA-mediated transport. However, in 1996 Takenouchi et al. [121] demonstrated that the bulkiness of the C-7 substituent and the hydrophobicity and bulkiness of the C-8 substituent, not the molecular hydrophobicity, was correlated with the activity of fluoroquinolones. In the meantime, many studies have been undertaken to show the effect of NorA in quinolone-resistant strains. For example, Schmitz et al. [122] examined the effect on MIC and IC50 values of ciprofloxacin, sparfloxacin and moxifloxacin in 102 clinical isolates. In about 50% of the strains, treatment with reserpine resulted in up to a fourfold decrease in the MIC values of ciprofloxacin and moxifloxacin and up to an eightfold decrease in the corresponding IC50 values, while in the other strains there was no visible effect. Sparfloxacin was unaffected by the addition of reserpine and has recently been shown to be a noncompetitive inhibitor of a hydrophilic dye [123]. Another study of Munoz-Bellido et al. [124] demonstrated in 47 clinical isolates that efflux can appear in clinical strains even in the absence of mutations in the genes usually involved in quinolone resistance, particularly in strains for which MICs are at or above the breakpoint.

In 1998 Sulavik and Barg [125] examined mutants of clinical isolates of methicillin-resistant and -susceptible *Staphylococcus aureus* with low-level resistance to ciprofloxacin. The comparison of generated first-step mutants and mutants with low-level ciprofloxacin resistance showed that in vitro selection did not alter the distribution into different classes of first-step mutants, with about 10% of the first-step mutants showing increased efflux.

In 1999 Aeschlimann et al. [126] examined the in vitro antibacterial activities and postantibiotic effects of levofloxacin, ciprofloxacin and norfloxacin in genetically related strains of *Staphylococcus aureus*. They used the wild-type and two NorA-mediated resistant strains previously described by Kaatz and Seo [116] (see above) for susceptibility testing and determination of time-kill curves and postantibiotic effect. It was found that the NorA inhibitors reserpine and omeprazole dramatically improve the activities of the more hydrophilic quinolones (ciprofloxacin and norfloxacin). These compounds may restore the activities of the quinolones by increasing intracellular drug concentrations, suggesting that the therapeutic use of efflux inhibitors may be a strategy to lower fluoroquinolone resistance. This is especially important because resistance due to increased efflux might allow the bacteria to survive in the time between contact with the quinolone and the development of resistance mutations in the target genes. This was proposed and shown by Markham et al. [127] and also by Beyer et al. [128], who developed an assay using reserpine to investigate fluoroquinolone efflux in *Streptococcus pneumoniae* and *Staphylococcus aureus*. Beyer et al. demonstrated the hydrophobic drugs trovafloxacin and moxifloxacin, with a bulky C-7 substituent, to be poor substrates of NorA and, as mentioned above, they found that the ease of selection of resistant mutant strains correlated well with efflux susceptibility.

Several studies of Neyfakh et al. [129], Zeller et al. [130] and Brenwald et al. [131] hinted at an efflux mechanism similar to the NorA efflux pump in *Streptococcus pneumoniae*. *Streptococcus pneumoniae* strains with a similar phenotype of resistance against ethidium bromide, fluoroquinolones and other transportable compounds and a possible elimination of this effect by reserpine and inhibitors of the proton motive force were discovered. In 1999 Gill et al. [132] identified the PmrA efflux pump, which can also be assigned to the MFS group and has a 24% amino acid homology to NorA.

In viridans streptococci and enterococci, it is highly likely that efflux pumps can be found as well. The role of efflux in resistance has already been demonstrated in viridans streptococci and enterococci. Lynch et al. [133] showed that, in *Enterococcus faecium* and *Enterococcus faecalis*, most strains pumped out norfloxacin and chloramphenicol. Meanwhile, it has been shown for enterococci and streptococci that reserpine is also able to lower the MIC of hydrophilic fluoroquinolones [133, 134]. However, the genes encoding those proteins have yet not been defined.

Conclusions

The increasing potency of the newer fluoroquinolones and the potential of NFQs offer hope that we can remain one step ahead of resistance development in vivo. At present, the structural modification of quinolones at the C-7 and C-8 positions to alter target specificity has yielded results. However, even the most potent of the current drugs, as we have seen, can select for resistance in vitro through target-site modification, active drug efflux, or both. In all probability, this will occur in clinical practice. How we address this, either through antibiotic policy or the introduction of ever newer drugs, remains to be seen. A new challenge for drug development – active efflux – has emerged in recent years, and it is imperative that it be overcome, as it threatens not only quinolones but unrelated drugs as well. Inhibitors of efflux may slow down the development of resistance or extend the life of current quinolones, much the same way beta-lactam inhibitors have prolonged the life of some beta-lactam agents. Although it is no easy task, given our current knowledge and the continual advances in drug design, proteomics and genomics, these challenges will be overcome – until the next one arises.

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