REVIEW ARTICLE

Drugs 1999; 58 Suppl. 2: 6-10
0012-6667/99/0002-0006/$02.50/0

Mode of Action of Fluoroquinolones

David C. Hooper

Division of Infectious Diseases, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts, USA

Abstract

The mode of action of quinolones involves interactions with both DNA gyrase, the originally recognised drug target, and topoisomerase IV, a related type II topoisomerase. In a given bacterium these 2 enzymes often differ in their relative sensitivities to many quinolones, and commonly DNA gyrase is more sensitive in Gram-negative bacteria and topoisomerase IV more sensitive in Gram-positive bacteria. Usually the more sensitive enzyme represents the primary drug target determined by genetic tests, but poorly understood exceptions have been documented.

The formation of the ternary complex of quinolone, DNA, and either DNA gyrase or topoisomerase IV occurs through interactions in which quinolone binding appears to induce changes in both DNA and the topoisomerase that occur separately from the DNA cleavage that is the hallmark of quinolone action. X-ray crystallographic studies of a fragment of the gyrase A subunit, as well as of yeast topoisomerase IV, which has homology to the subunits of both DNA gyrase and topoisomerase IV, have revealed domains that are likely to constitute quinolone binding sites, but no topoisomerase crystal structures that include DNA and quinolone have been reported to date.

Inhibition of DNA synthesis by quinolones requires the targeted topoisomerase to have DNA cleavage capability, and collisions of the replication fork with reversible quinolone-DNA-topoisomerase complexes convert them to an irreversible form. However, the molecular factors that subsequently generate DNA double-strand breaks from the irreversible complexes and that probably initiate cell death have yet to be defined.

Substantial new information regarding the mode of action of fluoroquinolones has been uncovered since the time of the 5th International Symposium on New Quinolones and Related Antibiotics in 1994. Particularly noteworthy have been the elucidation of dual drug targets within the bacterial cell, the 3-dimensional structures of several topoisomerases, conformational changes in the target enzymes and DNA associated with quinolone interaction, and the role of enzyme-DNA-quinolone complexes as barriers to DNA replication and transcription.

1. Dual Quinolone Targets

DNA gyrase was the first identified quinolone target, based on initial genetic studies with nalidixic acid-resistant mutants of *Escherichia coli* that had mutations mapping in what were subsequently shown to be the *gyrA*[1-3] and *gyrB*[3] genes encoding the enzyme's 2 subunits. The discovery of *E. coli* topoisomerase IV[4] revealed homology between the genes encoding its subunits, *parC* and *parE*, and *gyrA* and *gyrB*, respectively, with a particularly high conservation of amino acid sequences in the regions in which quinolone resistance mutations in *gyrA* and *gyrB* were localised.

Subsequently, purified topoisomerase IV of *E. coli* was shown to be inhibited by quinolones, although higher concentrations were required than for inhibition of purified DNA gyrase.[5] Proof of topoisomerase IV as another quinolone target *in vivo* came from studies in which first-step quinolone resistance mutations were found in *parC* (also termed *grlA*) in *Staphylococcus aureus*[6-8] and second-step *parC*[9] and *parE*[10,11] mutations were shown to cause increments in quinolone resistance in the presence of *gyrA* mutations.

For many other Gram-negative bacteria, as with *E. coli*, first-step quinolone resistance mutations occur in *gyrA*, or less commonly *gyrB*.[12] In contrast, for many Gram-positive bacteria, as with *S. aureus*, first-step quinolone resistance mutations occur in *parC*, or less

commonly *parE*. The primary drug target enzyme as defined by first-step resistance mutations thus commonly differs between Gram-positive and Gram-negative bacteria. Furthermore, resistance mutations in the subunits of the secondary drug target enzyme (topoisomerase IV for *E. coli* and DNA gyrase for *S. aureus*) exhibit little or no resistance phenotype except in the presence of resistance mutations in the primary target enzyme.[9,10,13] These differences appear to result from the relative sensitivities of DNA gyrase and topoisomerase IV to particular quinolones in a given organism.

For *E. coli*, purified DNA gyrase is more sensitive to many quinolones than purified topoisomerase IV, and conversely in *S. aureus* purified topoisomerase IV is the more sensitive of the 2 enzymes.[14] Thus, the more sensitive enzyme generally determines the primary drug target for a given organism. Interaction of a quinolone with the primary drug target thus determines cell susceptibility independently of the sensitivity of the secondary drug target.

There have, however, been at least two notable exceptions to this pattern. In *Streptococcus pneumoniae*, a Gram-positive pathogen, sparfloxacin and clinafloxacin select first-step *gyrA* mutations rather than *parC* mutations.[15,16] Furthermore, contrary to expectations, purified *S. pneumoniae* DNA gyrase was found to be less sensitive to sparfloxacin and clinafloxacin than purified topoisomerase IV.[17,18] The explanation for the apparently anomalous behaviour of sparfloxacin and clinafloxacin is unclear. It is possible to speculate that the greater hydrophobicity of sparfloxacin relative to that of many other quinolones might result in alterations in its distribution to cellular compartments and that DNA gyrase may thereby be exposed to higher drug concentrations *in vivo* than is topoisomerase IV. Further data are needed.

The recognition of dual drug target enzymes also has important implications for the development of resistance. For resistance mutations in the primary target enzyme, the increment in resistance may be limited by the level of sensitivity of the secondary target enzyme, which becomes the more sensitive enzyme when the primary target is resistant.

This scheme implies that for different quinolones the level of resistance conferred by a mutation in the primary target enzyme would decrease as the level of drug sensitivity of the secondary target approaches that of the primary target. Furthermore, it implies that concurrent dual mutations in both target enzymes would be required for resistance resulting from target alteration for any quinolone that had equal potency against DNA gyrase and topoisomerase IV. This limit appears to be approximated with clinafloxacin, a quinolone under development, in *S. pneumoniae*.[16] Mutants of *S. pneumoniae* selected with clinafloxacin occur at exceedingly low frequency ($<8 \times 10^{-10}$ at 2-fold MIC), and those *gyrA* mutations selected in first-step mutants result in a minimal increment in clinafloxacin resistance (2-fold). Later step mutants with mutations in both *gyrA* and *parC*, however, exhibit substantial resistance.

Thus, some quinolone structures appear to target both DNA gyrase and topoisomerase IV to a similar degree. It is not yet clear if equivalent dual targeting by a quinolone in one species predicts similar behaviour in other species. In at least 3 species – *Mycobacterium tuberculosis*, *Helicobacter pylori*, and *Treponema pallidum* – dual targeting will not be possible, since these organisms appear to lack genes for topoisomerase IV, as determined by complete genome sequencing.[19-21]

2. Interactions of Quinolones with Topoisomerase-DNA Complexes

There has been recent additional information about the 3-dimensional structures of the gyrase A and B subunits and the related yeast enzyme topoisomerase II, the domains of which have homology to GyrB (N terminus) and GyrA (C terminus).[22-24] In addition, the recently resolved crystal structure of a 59-Kd fragment of GyrA[23] supports the similarities in protein structure between the bacterial and yeast enzymes but also identifies a different conformational state which is likely to represent a different step in the enzymatic catalytic cycle.[25]

For both enzymes, the positions of amino acids that affect quinolone sensitivity (i.e. determine resistance when mutant) are clustered in alpha helices near to the active-site tyrosine, which is involved in DNA breakage. In the GyrA fragment, these amino acids are located along a positively charged surface to which DNA may bind based on structural modelling. Thus, this site might be hypothesised to be the region at which quinolones bind to the enzyme-DNA complex.

This hypothesis is supported by earlier data showing that DNA gyrase-DNA complexes reconstituted with resistant mutant GyrA protein bind quinolones less well.[26] This potential quinolone-binding site in GyrA, however, is distant from the regions of the yeast enzyme that correspond to the amino acids in GyrB involved in quinolone sensitivity.[22] Thus, quinolone resistance caused by mutations in *gyrB* either might have a mechanism different from alteration in a primary drug binding site or might result from alteration in another quinolone binding site revealed in other conformations of DNA gyrase in which these regions

of GyrB are in proximity to the proposed site of quinolone binding in GyrA.

It is noteworthy that recent crystal structures of yeast topoisomerase II reveal that the regions homologous to those of GyrA and GyrB that affect quinolone action may be in proximity to each other in newly identified enzyme conformations.[27] Crystal structures of complexes of a quinolone, DNA, and fragments of GyrA and GyrB (or ParC and ParE) will be needed to address these issues definitively.

Specific binding of quinolones is known to require both DNA and topoisomerase,[26,28] and thus it is the enzyme-DNA complex that is the target of this class of drugs. Several lines of evidence indicate that interaction of quinolones with a complex of DNA and a type II topoisomerase affects DNA binding and DNA structure as well as topoisomerase conformation. Furthermore, quinolone binding to the enzyme-DNA complex can be dissociated from quinolone-induced DNA cleavage by topoisomerase.

Norfloxacin has been shown to stabilise the interaction of DNA with topoisomerase IV, and this stabilisation can occur in the absence of DNA cleavage and ATP,[29] suggesting that quinolones can act at a step preceding DNA strand cleavage. Ciprofloxacin has also been shown to bind to complexes of DNA and a mutant DNA gyrase that cannot carry out DNA cleavage[30] and to promote conformational changes in the enzyme in the absence of DNA cleavage.[31]

These conformational changes alter the rate of ATP hydrolysis, and this quinolone-characteristic rate was used to monitor the kinetics of drug binding and DNA cleavage.[32] The rate of ATP hydrolysis is altered rapidly by ciprofloxacin, but DNA cleavage occurs at a much slower rate, allowing dissociation of the 2 processes.

Enoxacin was also shown to convert complexes of DNA and DNA gyrase slowly to a stable cleavable complex in the absence of ATP. Although this stable form was detected by its ability to form DNA cleavable complexes upon addition of a protein denaturant, enoxacin binding and the ability to promote cleavage were reversible, indicating that irreversible strand cleavage was not a requirement for complex stabilisation.[33]

Thus, quinolones appear to stabilise binding of DNA to topoisomerase complexes and to promote enzyme conformational changes prior to, and without a requirement for DNA cleavage.

DNA structure itself is also altered by norfloxacin binding to topoisomerase IV-DNA complexes, as revealed by altered reactivity with $KMnO_4$, which oxidises thymine residues that can then be cleaved by piperidine.[29] This quinolone-induced distortion of DNA occurs in the absence of DNA strand cleavage and is greater than that seen in the absence of topoisomerase IV. These findings are consistent with a model in which quinolones bind in proximity to a DNA binding site on topoisomerase IV, if not to the DNA itself, within the topoisomerase IV drug binding site, thereby distorting or stabilising a distortion of DNA in the complex.

3. Interactions of Quinolone-Topoisomerase-DNA Complexes with DNA Replication Forks and RNA Polymerase

Quinolones inhibit DNA synthesis, and at higher concentrations, RNA synthesis as well. These effects are mediated by the ability of quinolones to stabilise complexes of DNA and type II topoisomerases. Inhibition of DNA synthesis can occur from quinolone interactions with both DNA gyrase and topoisomerase IV *in vivo*.[34]

This process has been studied *in vitro* with purified DNA replication systems. Quinolone-stabilised complexes of DNA and topoisomerase IV are reversible with the addition of edetic acid (EDTA). Collision of the complex with an active DNA replication complex, however, converts the complex to a form that is not reversed by EDTA,[35] but does not generate DNA cleavage. This collision also blocked progression of the replication fork. A mutant topoisomerase IV that lacks DNA cleavage capability bound DNA but was unable in the presence of a quinolone to block progression of the replication complex. Thus, complexes of DNA, quinolone, and active topoisomerase IV appear to form physical barriers to DNA replication. Although the interaction between replication and topoisomerase complexes converts the DNA-topoisomerase-quinolone complex to a more stable form, this interaction alone appears to be insufficient to generate double-strand DNA breaks, which are thought to be necessary for the bacterial lethality produced by quinolones.

If other cellular components, such as those mediating an abortive DNA repair process, are involved in a second step to generate DNA cleavage, then the stabilised 'pre-cleavage' complexes generated by the *in vitro* DNA replication system may provide an assay for such an activity in cell extracts. Identification of the molecule(s) effecting DNA cleavage by the stabilised 'pre-cleavage' complexes might provide insight into the long-elusive molecular determinants of quinolone bactericidal activity.

In vivo, inhibition of DNA synthesis by interaction with DNA gyrase occurs rapidly,[36] but inhibition at-

tributable to interaction with topoisomerase IV occurs with some delay.[34] This difference is thought to relate to differences in the localisation of DNA gyrase and topoisomerase IV on the bacterial chromosome.

DNA gyrase is thought to be localised in front of DNA replication forks, so that collisions occur soon after quinolone-gyrase-DNA complexes form. Topoisomerase IV, in contrast, is proposed to be localised behind DNA replication forks and thus does not collide with the fork until a subsequent cycle of DNA replication.

Only recently has it been possible to localise topoisomerase IV and DNA gyrase within intact cells.[37] Gyrase subunits were localised to the nucleoid and, in contrast, ParC of topoisomerase IV exhibited a bipolar localisation that was dependent on functional ParE. The relationship of these localisations to DNA replication complexes, however, has not yet been determined.

Quinolone-DNA gyrase-DNA complexes have also been shown to block the passage of RNA polymerase and to lead to premature termination of transcription in an *in vitro* transcription system.[38] As with the block to DNA replication, a mutant DNA gyrase incapable of DNA cleavage was also unable to block transcription.[30]

4. Areas for Future Investigation

Considerable progress has been made in elucidating the details of the way that quinolones interact with their target enzymes and inhibit DNA synthesis. Remaining areas for which further data are needed include defining the site of binding of quinolones to the DNA-topoisomerase complex, the conformation of the enzyme in which this site is formed, and conformational changes in topoisomerase and DNA induced by quinolone binding.

In addition, there remains a need to determine the molecular factor(s) that generate(s) DNA double-strand breaks *in vivo* after quinolone interaction with the DNA-topoisomerase complex and the role of this factor(s) in quinolone bactericidal activity. The rate of recent progress provides hope that even this last need, which has been the most refractory to progress, will be met in the near future.

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Correspondence and reprints: *David C. Hooper,* M.D., Division of Infectious Diseases, Massachusetts General Hospital, 55 Fruit Street, Boston, MA 02114-2696, USA.
E-mail: dhooper@partners.org