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Leading articles

The molecular basis of quinolone action

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The quinolones are a group of antibacterial agents based on or resembling the 4-oxo-1, 4-dihydroquinoline skeleton. The first member of this group to be synthesized was nalidixic acid (Figure) (Lesher *et al.*, 1962), which showed activity against Gram-negative bacteria. Since then a very large number of quinolones have been synthesized (see for example, Rádl, 1990; Chu & Fernandes, 1991). Some of these have antibacterial potencies 1000 times greater than that of nalidixic acid and are active against both Gram-negative and Gram-positive organisms. Many of the new drugs have a fluorine atom at position 6 of the quinolone structure (e.g. norfloxacin, ciprofloxacin, sparfloxacin; Figure), which significantly enhances antibacterial potency.

The intracellular target of the quinolones is DNA gyrase, the enzyme which catalyses the negative supercoiling of DNA in bacteria (Gellert *et al.*, 1976). Gyrase consists of two proteins, A and B, of molecular weights 97 and 90 kDa respectively, which are encoded by the *gyrA* and *gyrB* genes of *Escherichia coli*; the active enzyme is a A_2B_2 complex (for a review see Reece & Maxwell, 1991*a*). The mechanism of DNA supercoiling by gyrase involves the passage of a segment of DNA through a double-stranded DNA break held open by the enzyme. Catalytic supercoiling requires the hydrolysis of ATP. The A subunits of gyrase are involved in the DNA breakage and resealing aspects of supercoiling, while the B subunits are responsible for ATP hydrolysis. Quinolone drugs can completely inhibit the DNA supercoiling reaction, apparently by interrupting the DNA breakage and resealing steps. Direct evidence for this comes from experiments in which reactions involving gyrase, DNA and a quinolone drug are terminated by the addition of sodium dodecyl sulphate. These results show double-strand cleavage of the DNA (with a 4-base stagger between the break sites) and covalent attachment of the gyrase A subunits to the newly-formed 5′-phosphate groups (Gellert *et al.*, 1977; Morrison & Cozzarelli, 1979). Specifically a phosphate ester is formed between the hydroxyl group of Tyr122 and 5′-terminus of the DNA (Horowitz & Wang, 1987).

In addition to catalysing negative supercoiling, gyrase can also relax both negatively and positively supercoiled DNA, unknot DNA, and catenate and decatenate double-stranded DNA circles. All these reactions involve the DNA breakage and strand-passage processes, and are therefore sensitive to inhibition by quinolones. Another well known group of antibacterial agents, the coumarins, also act on DNA gyrase, but in an entirely different way to the quinolones, by inhibiting the ATPase reaction (for a review, see Reece & Maxwell, 1991*a*). Thus the ATP-requiring DNA supercoiling reaction is sensitive to coumarins, while the ATP-independent relaxation of negative supercoils is unaffected by these drugs.

Despite extensive research work both on the quinolones and on DNA gyrase, the molecular details of the interaction of these drugs with the enzyme and how this leads to cell death are incompletely understood. It is convenient to divide the events leading to bacterial killing by quinolones into four stages: entry of the drugs into the cell; interaction of the drugs with gyrase and DNA; the cytotoxic event; cell death. This article deals with the second and third of these stages; other aspects of quinolone action are discussed elsewhere (Wolfson & Hooper, 1990; Piddock, 1991).

Is is now widely accepted that DNA gyrase is the intracellular target of quinolones. The most compelling evidence in support of this is the existence of single-point mutations in the gyrase genes which confer high levels of resistance to the quinolone drugs. Examples of such mutations are given in the Table. Several important points arise from these data. Firstly, the mutations more frequently map to *gyrA* than to *gyrB*. Indeed only two mutations have been found to map to *gyrB*, and one of these (Lys447 → Glu) actually shows hypersensitivity to pipedimic acid, a non-fluorinated quinolone (Yamagishi *et al.*, 1981, 1986; Yoshida *et al.*, 1991). It should be noted however, that mutations can also be found to

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Table. Quinolone-resistance mutations of *E. coli* DNA gyrase

Subunit	Amino acid change	MIC (mg/L)[b] nalidixic acid	MIC (mg/L)[b] ciprofloxacin	Reference
(wild-type)	—	3·13	0·0125	1[c]
A	Ala67 → Ser	25	0·05	1
A	Gly81 → Cys	50	0·1	2
A	Ser83 → Ala[a]	ND	0·06	3
A	Ser83 → Leu	400	0·39	1,4
A	Ser83 → Trp	400	0·39	1,4,5
A	Ser83 → Tyr[a]	ND	ND	6
A	Ala84 → Pro	25	0·1	2
A	Asp87 → Asn	200	0·2	2
A	Asp87 → Val	31	0·06	4
A	Gln106 → Arg	ND	0·05	3
A	Gln106 → His	12·5	0·05	1
B	Asp426 → Asn	50	0·1	7
B	Lys447 → Glu	50	0·1	7

[a]Mutations generated by site-directed mutagenesis.
[b]Minimum inhibitory concentration.
[c]References: 1, Yoshida *et al.* (1988); 2, Yoshida *et al.* (1990); 3, Hallett & Maxwell, (1991); 4, Oram & Fisher (1991); 5, Cullen *et al.* (1989); 6, Willmott & Maxwell (unpublished observation); 7, Yamagishi *et al.*, (1986); 8, Yoshida *et al.* (1991).
ND, Not determined.

map outside the gyrase genes, often in genes encoding outer membrane proteins and thus presumably affecting quinolone uptake (Piddock, 1989; Hooper & Wolfson, 1990). Secondly, the gyrase A protein mutations are closely clustered in a region of ~40 amino acids (Table). This region occurs in the N-terminal domain of the A protein (Reece & Maxwell, 1989, 1991*b*), which is responsible for the DNA breakage/resealing reactions of gyrase. Indeed enzyme consisting of the 64 kDa N-terminal domain of the A protein and the intact B protein is as proficient at carrying out quinolone-induced cleavage as is the intact enzyme (A_2B_2) (Reece & Maxwell, 1991*b*). The region containing the quinolone-resistance mutations is close in the linear amino acid sequence to Tyr122, which is involved in the formation of covalent bonds between DNA and protein. It should be noted that Tyr122 may be distant from this region in the final folded structure of the protein. Thirdly, it has been found that the amino acid residue most frequently mutated in spontaneous *gyrA* mutations is Ser83. Mutation of this residue to Leu or Trp confers very high levels of resistance (Table).

The occurrence of such mutations in *gyrA* would naturally lead to the expectation that the quinolones bind directly to the A subunit of gyrase. However, studies by Shen and co-workers have led to a somewhat different conclusion. Their initial data showed no binding to either the gyrase A or B proteins, or to the intact enzyme (A_2B_2), but significant binding to DNA (Shen & Pernet, 1985). Further work showed that gyrase could stimulate the binding of quinolones to double-stranded DNA (Shen *et al.*, 1989*a*). These and other data have been incorporated into a model for the interaction of quinolones with gyrase and DNA (Shen *et al.*, 1989*b*). In this model gyrase cleaves double-stranded DNA with a 4-base stagger, as described above. The exposed single-strand regions are proposed to constitute the binding sites for the drugs, which interact with the bases *via* hydrogen bonding to the 3-carboxy and 4-oxo groups common to nearly all quinolones (Figure). Drug binding is thought to be co-operative, with at least four molecules binding per site, associating with each other by ring stacking and hydrophobic interactions. Interaction with gyrase is proposed to occur *via* the group at the C-7 position of the quinolone (Figure).

Work from a number of other laboratories both supports and refutes this model (reviewed in Reece & Maxwell, 1991*a*) and it remains a controversial and thought-provoking issue. One potential difficulty with this model is the claim that interaction with gyrase is *via* the C-7 group of the drug, a position that is highly variable in quinolones (see Figure). Given the usual high degree of specificity in protein-

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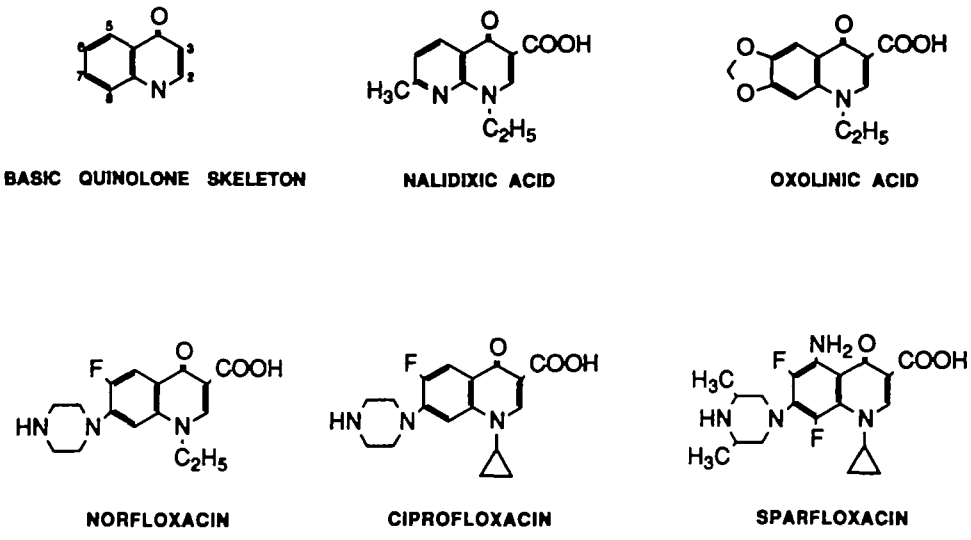

Figure. Structures of quinolones.

ligand interactions, this proposal is somewhat surprising. A more logical suggestion would be hydrogen bonding between the 3-carboxy and 4-oxo groups on the drug and hydrogen-bond donors on the enzyme. Candidates for such hydrogen bond donors can be found among the residues given in the Table (e.g. Ser83, Gln106).

Recent data from my laboratory support such a suggestion (Willmott, C. J. R. & Maxwell, A., unpublished data). Using the technique of rapid gel filtration we have found binding of norfloxacin to the gyrase-DNA complex but not to the protein or to DNA alone. The DNA in this case is a 147 bp fragment containing the preferred gyrase cleavage site from plasmid pBR322. Replacement of the gyrase with enzyme consisting of A protein bearing the mutation Ser83 → Trp (Table) leads to considerable reduction in binding to the protein-DNA complex. Such a result suggests that Ser83 interacts directly with the quinolone drug.

Thus it seems that the primary binding site for the quinolone drugs is the gyrase-DNA complex, with contacts between the drug and both the DNA and the protein. Whether the contacts are just with the A protein or with the B protein as well is not clear. It is possible that mutations to the B protein exert their effects *via* interactions with the A protein. It is interesting to compare the interaction of quinolone drugs with gyrase with the interaction of antitumour drugs with DNA topoisomerase II (the close relative of gyrase in eukaryotes). Topoisomerase II drugs such as doxorubicin, amsacrine and teniposide are proposed to intercalate between the base pairs of the DNA at the enzyme's active site (Capranico, Kohn & Pommier, 1990; Pommier *et al.*, 1991), again suggesting a drug-enzyme-DNA ternary complex. With both gyrase/quinolones and topoisomerase II/anti-tumour drugs the exact details of the ternary complex are largely a matter of speculation. Clearly this is an area where high resolution structural data is much needed. The recent crystallization of the N-terminal domain of the gyrase A protein is a step in this direction (Reece *et al.*, 1990).

After the formation of the quinolone-gyrase-DNA complex an event must occur which will initiate the process of cell death. *A priori* this could be the inhibition of supercoiling by gyrase. However, two observations argue against this idea. Hane & Wood (1969) found that nalidixic acid-sensitive alleles of *gyrA* were dominant over nalidixic acid-resistant alleles. Thus, introduction of a plasmid bearing a wild-type *gyrA* gene into a strain encoding a *gyrA* gene conferring quinolone resistance leads to a sensitive phenotype (e.g. Nakamura *et al.*, 1989). The other observation has been termed the 'MIC paradox' and concerns the fact that the concentration of quinolone required to inhibit DNA supercoiling by gyrase *in vitro* or *in vivo* exceeds by a factor of 10–100 that required to inhibit bacterial growth (Gellert *et al.*, 1977; Domagala *et al.*, 1986; Zweerink & Edison, 1986). Such a result is unexpected as the

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isolated target (gyrase) would be predicted to be more not less sensitive than the intact cell. One explanation for the latter observation is that other reactions of gyrase, such as relaxation and decatenation, may be more sensitive to quinolones than is supercoiling. However, Hallett & Maxwell (1991) showed that both the DNA relaxation and the decatenation reactions were no more sensitive to inhibition by quinolone drugs than was DNA supercoiling.

Hence explanation other than inhibition of the enzymic reactions of gyrase is required for the inhibitory effect of quinolones on bacterial cell growth Kreuzer & Cozzarelli (1979) advanced the 'poison hypothesis', namely that the interaction of a quinolone with gyrase in the cell converts it into a poison. This poison might be the quinolone-gyrase-DNA complex, which forms a barrier to the passage of polymerases. Indeed filter-binding experiments show that this ternary complex is more stable than the gyrase-DNA complex (Higgins & Cozzarelli, 1982). Such a proposal can explain both the above observations. In a *gyrA* partial diploid the wild-type enzyme will still form the ternary complex irrespective of the copy number of the quinolone-resistant allele. The MIC paradox can be explained by the polymerase block occurring when only a small percentage of the intracellular gyrase molecules have been bound to quinolone. This would be at a concentration well below that at which significant inhibition of supercoiling would be seen.

Recent experiments from this laboratory lend some support to these ideas. Transcription by T7 RNA polymerase *in vitro* has been found to be unaffected by the presence of either gyrase or quinolone drugs. However, the presence of both gyrase and quinolone leads to premature termination of transcription (Willmott, C. J. R., Eperon, I. C. & Maxwell, A., unpublished data).

The collision between polymerases and complexes made up of anti-tumour drugs, eukaryotic topoisomerases and DNA, has also been suggested as a mechanism for the cytotoxic effects of anti-tumour drugs (Liu, 1989). Hsiang, Libou & Liu (1989) have provided evidence that collision between moving replication forks and a camptothecin-topoisomerase I-DNA complex results in fork arrest and possibly fork breakage.

One surprising inference from the above is that polymerases can normally pass bound gyrase (and other topoisomerase) molecules. Precedents for this are found in other systems. For example, the bacteriophage SP6 polymerase and mammalian RNA polymerase II have been shown to transcribe through nucleosomes *in vitro* (Lorch, LaPointe & Kornberg, 1987; Losa & Brown, 1987). In addition, T4 replication forks can pass nucleosomes in an *in vitro* replication system (Bonne-Andrea, Wong & Alberts, 1990). It remains to be determined in the case of gyrase whether the protein is displaced during transcription or replication.

In summary, the available experimental evidence can be interpreted to suggest that quinolones interact with both gyrase and DNA to form a stable ternary complex. This complex may well be the lesion which initiates cell death, possibly by forming a barrier to the passage of DNA and RNA polymerase molecules in the cell. Clearly a great deal more experimental work is required to elucidate the details of these processes.

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Lack of quinolone-induced arthropathy in children

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The newly developed derivatives of nalidixic acid, the fluoroquinolones, have been studied extensively in the laboratory and in clinical trials. Compared with nalidixic acid the new quinolones exhibit an expanded antimicrobial spectrum, greatly enhanced bactericidal activity, and substantial pharmacokinetic advantages. Numerous clinical studies have proved the efficacy and safety of these compounds for various infections in adult patients.

Many experts have advised against performing studies on the efficacy and safety of these promising agents in children because of potential adverse effects, limited activity against streptococci, and lack of adequate pharmacokinetic evaluation. In particular, the risk of cartilage toxicity in weight-bearing joints, which was observed in experiments with some young animals whose skeletal growth was incomplete, was thought to represent a contraindication for the use of quinolones in paediatric patients.

When administered to immature animals (dogs, rabbits, rats, marmosets, pigs) all the quinolones studied—older and newer derivatives—caused arthropathic effects in major, usually weight-bearing synovial joints (Gough *et al.*, 1979; Schlüter, 1986, 1989; Christ, Lehnert & Ulbrich, 1988; Burkhardt *et al.*, 1990; Stahlmann *et al.*, 1990). This arthropathy evolves within days to weeks and is characterized by cartilage toxicity accompanied by non-inflammatory joint effusion. Histopathological findings are localized blister formation and erosions in joint cartilage (Gough *et al.*, 1979; Schlüter, 1986; Christ *et al.*, 1988). Clusters of chondrocytes are found, indicating attempted cartilage repair. Recently, electron microscopic examination of such cartilage showed necrotic chrondrocytes and dissolution of matrix (Burkhardt *et al.*, 1990; Stahlmann *et al.*, 1990). These quinolone-induced cartilage lesions are usually irreversible, and the reduced quality of cartilage may promote degeneration, including arthropathia deformans. If clinically symptomatic, such arthropathy in animals manifests as acute arthritis, including limp and swelling. These toxic effects are dose-dependent, but occur at different dosages in different species. Moreover, animal cartilage toxicity varies between quinolone compounds: this could be ascribed to either true heterogeneity of arthropathogenicity or differences in pharmacokinetics. In all these animal experiments the older compounds (e.g. pipedimic acid, nalidixic acid) showed substantially greater arthropathogenicity than the fluoroquinolones.

The pathogenesis of quinolone-induced arthropathy in animals remains unexplained. The primary target of all quinolones is a bacterial DNA gyrase (topoisomerase II), an essential bacterial enzyme for DNA replication and certain aspects of transcription, DNA repair, recombination, and transposition (Smith, 1986). Although it is generally accepted that the fluoroquinolones are specific for prokaryotic enzymes, there are reports of in-vitro experiments indicating some inhibition of eukaryotic DNA replication (Castora, Vissering & Simpson, 1983; Gootz, Barrett & Sutcliffe, 1990). In immature rats dosed with ofloxacin, chondrocytes of the intermediate zone showed a transient decrease and subsequent increase in uptake of tritiated thymidine (Kato & Onodera, 1988). This observation could reflect early depression of DNA synthesis by the drug followed by reparative reaction of the chondrocytes. The hypothesis that quinolones might inhibit mitochondrial DNA in immature, metabolically active chondrocytes of certain animal species requires further investigation.

Because of the obvious advantages of the fluoroquinolones and the absence of joint pathology in follow-up studies of children treated with nalidixic acid (Schaad & Wedgwood, 1987; Adam, 1989), many paediatricians have started to prescribe these antibacterial agents for some of their patients on a compassionate use basis. By early 1992, published data on fluoroquinolone use in children included over 1000 pre-pubertal patients (Black *et al.*, 1990; Cheesbrough *et al.*, 1991; Chysky *et al.*, 1991; LeBel, 1991; Schaad, 1991). These studies report good to excellent efficacies, and usually mild and always revers-

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