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Interactions between fluoroquinolones, Mg^{2+}, DNA and DNA gyrase, studied by phase partitioning in an aqueous two-phase system and by affinity chromatography

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Abstract

The primary target of fluroquinolones has been identified as the enzyme DNA gyrase, but the mechanism of action of these antibacterial agents is still a matter of controversy. Using partitioning in aqueous polyethylene glycol (PEG)–dextran systems, the affinities of several fluoroquinolones for DNA were determined with accuracy and at equilibrium. It was proved that the binding was strongly dependent on the ability of the drugs to bind Mg^{2+}, with K_A values of about 40 000 l mol^{-1}, but was poorly related to the antibacterial activity [minimal inhibitory concentration (MIC) and gyrase inhibition]. Using affinity chromatography on immobilized fluoroquinolone, it was shown that DNA gyrase was unable to bind fluoroquinolones in the absence of DNA, but that a DNA–quinolone–gyrase complex was formed in the presence of Mg^{2+}.

1. Introduction

Fluoroquinolones (FQ) are antimicrobial agents widely used against various infections. Their primary target has been identified as the enzyme DNA gyrase (EC 5.99.1.3) [1,2], as fluoroquinolones are able to inhibit isolated gyrase [3] and as gyrases isolated from resistant organisms are resistant to fluoroquinolones [4]. DNA gyrase consists of two A and two B subunits (the products of the *gyrA* and *gyrB* genes, respectively). Since the first model proposed by Shen and Pernet [5] for the mechanism by which quinolones inhibit gyrase, this group have refined their model [6], which implies binding of the quinolone to DNA. Palù *et al.* [7] pinpointed the role played by Mg^{2+} ion in the DNA–quinolone interaction, and recently the role of DNA gyrase in the interaction was examined [8,9]. The important role played in antimicrobial activity of quinolones by Mg^{2+}, which impairs both oral absorption [10,11] and uptake by bacterial cell [12], prompted us to measure the affinity of Mg^{2+} for several fluoroquinolones. Having the K_D values of the Mg^{2+}–FQ complexes at hand, and having proved that Mg^{2+} was situated between the ketone and the carboxylate of quinolones [13], we decided to examine the effects of magnesium on the FQ–DNA interaction. We investigated whether the affinity of a quinolone for magnesium was determinant in the FQ–DNA interaction, and whether the affinity of a fluoroquinolone for DNA in the presence of magnesium was related to its inhibitory activity against DNA gyrase-catalysed supercoiling. The way in which DNA gyrase modified these interactions was also investigated.

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Most studies on interactions between an enzyme and its substrates or inhibitors have been carried out using equilibrium dialysis. In the present instance, the major limitation of this technique would be undesirable interactions between DNA and the dialysis membrane. Moreover, the size of both enzyme and substrate precluded the use of membranes. This problem could be overcome by using chromatography, but such experiments would not be performed under equilibrium conditions. Therefore, we decided to use aqueous two-phase partitioning [14,15], where the interface between the two phases plays the role of the separating membrane [16,17]. Some assays were performed using affinity chromatography on a resin coated with immobilized quinolone.

2. Experimental

2.1. Chemicals

Pefloxacin, sparfloxacin and RP 65279 were gifts from Rhône-Poulenc Rorer (Vitry sur Seine, France). [^{14}C]Pefloxacin and [^{14}C]sparfloxacin, from Rhône-Poulenc Rorer, were used at a specific activity of 40 μCi/mol. Compounds named BMY were donated by Bristol–Myers Squibb France (Marne la Vallée, France). These compounds have been described in refs. 18 and 19: BMY 33315, 42230, 43261 and 40062 are compounds 32, 33, 35 and 36, respectively, in ref. 18 and BMY 180820 and 180821 are compounds 39 and 43, respectively, in ref. 19. Ciprofloxacin was provided by Bayer France (Paris la Défense, France). Pefloxacin ethyl ester and norfloxacin methyl ester were synthesized in our laboratory using ethanol or methanol and thionyl chloride [20].

Dextran T500 and epoxy-activated Sepharose were obtained from Pharmacia France (Saint Quentin en Yvelines, France), polyethylene glycol (PEG) 6000 from Touzart et Matignon (Vitry sur Seine, France), Indubiose A37 was from IBF (Villeneuve la Garenne, France), covalently closed supercoiled plasmid pBR322 from Boehringer (Meylan, France) and DNA "highly polymerized" from calf thymus and DNA from *Escherichia coli* from Sigma (Saint-Quentin-Fallavier, France).

2.2. Minimal inhibitory concentrations (MICs)

Bacteria were routinely grown in trypticase soy broth (TSB) from Biomérieux (Marcy l'Etoile, France). MICs were determined by using twofold dilutions of antibiotic in TSB, to which 10^6 colony-forming units of bacteria were added. Cultures were incubated for 18 h at 37°C.

2.3. DNA gyrase purification and supercoiling assays

Purification of gyrase from *E. coli* K12 J53 and assays for inhibition of supercoiling using plasmid pBR322 were performed as described earlier [21]. The inhibition was expressed as MED (minimal effective dose, the minimal fluoroquinolone concentration able to affect supercoiling), as described previously [21].

2.4. Phase system and partitioning experiments

A 6% (w/w) dextran–4% (w/w) PEG mixture was made in buffer consisting of 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES)–50 m*M* KOH (pH 7.5)–20 m*M* KCl–0 or 5 m*M* $MgCl_2$ as specified. The mixture was left overnight at 4°C, where separation occurred, and the phases were separated and kept at 4°C.

Partitioning experiments were carried out in glass test-tubes. Fluoroquinolone was added to 1.1 ml of the dextran phase up to 4 μM, then DNA was added and its concentration was varied from 16.4 to 1640 μM of base. In another series of experiments, the concentration of DNA was fixed at 57 μM of base, and the fluoroquinolone concentration varied from 0.9 to 120 μM (final concentration). Incubation was carried out for 10 min at 20°C, then 1.1 ml of PEG phase was added and the phases were mixed by inversion and separated by centrifugation for 10 min at 2000 *g*. The volume of added reagents was constant (total volume 200 μl for a 2.2 ml total reaction volume). The dosage of DNA in

the phases was carried out either by measuring the absorbance at 260 nm ($\epsilon_{260\,nm}$ = 6600 mol base l^{-1} cm^{-1}) or using ethidium bromide [22]; fluoroquinolones were assayed using radio-labelled molecules when available, or by fluorescence (Hitachi F-2000 Spectrofluorimeter, supplied by Braun, Les Ulis, France). Standard dosage curves were established at the maximum wavelength of each compound in the PEG phase. When DNA was present, dosage cannot be performed in the dextran phase because of modification of the fluorescence of the quinolone on binding to DNA. The partition coefficient was the ratio of the concentration of material in the top phase to that in the bottom phase.

2.5. Affinity chromatography

Norfloxacin (the desmethyl analogue of pefloxacin, with an NH instead of an NCH_3 group on the piperazine) was immobilized on epoxy-activated Sepharose following the supplier's recommendations (see Fig. 1). Briefly, 2 g of epoxy-activated Sepharose were reacted with 200 mg of norfloxacin in 6 ml of 0.3 *M* carbonate buffer (pH 9.5) for 17 h at 37°C. Ethanolamine (0.55 ml) was then added and reacted for 4 h at 37°C. After thorough washings [carbonate buffer (pH 9.5), water, acetate buffer (pH 4), water, 5 *M* urea, water], a 1-ml column was packed and equilibrated with buffer E [Tris–50 m*M* HCl (pH 7.5)–20 m*M* KCl–2 m*M* $MgCl_2$].

DNA (100 μl of a 4 mg ml^{-1} solution, calf thymus or *E. coli*), gyrase (500 μl of a 40 μg ml^{-1} solution) or mixtures of both were added to the column, and after 1 h of contact at 4°C under gentle rocking, to ensure good contact of the reagents, washing was performed with buffer E until there was no more absorbance at 260 nm (DNA) or no more fluorescence (protein, excitation at 280 nm, emission at 340 nm). Elution was then carried out as described in the figure captions, followed by a washing with 4 *M* NaCl to regenerate the column before re-equilibration.

3. Results

3.1. Affinity of fluoroquinolones for DNA

Under the experimental conditions used, high-molecular-mass DNA totally partitioned into the dextran-rich phase, whereas the fluoroquinolones have partition coefficients between 0.9 and 1.3. Table 1 presents the MICs for *E. coli*, MEDs for *E. coli* gyrase and the affinity (expressed as nK_A for calf thymus DNA of the assayed fluoroquinolones, for the experiments carried out with fixed fluoroquinolone concentration, or as the K_A for calf thymus DNA of the quinolones assayed with fixed DNA concentrations). Some experiments were carried out using *E. coli* double-stranded DNA. As the results were the same as with calf thymus DNA, the

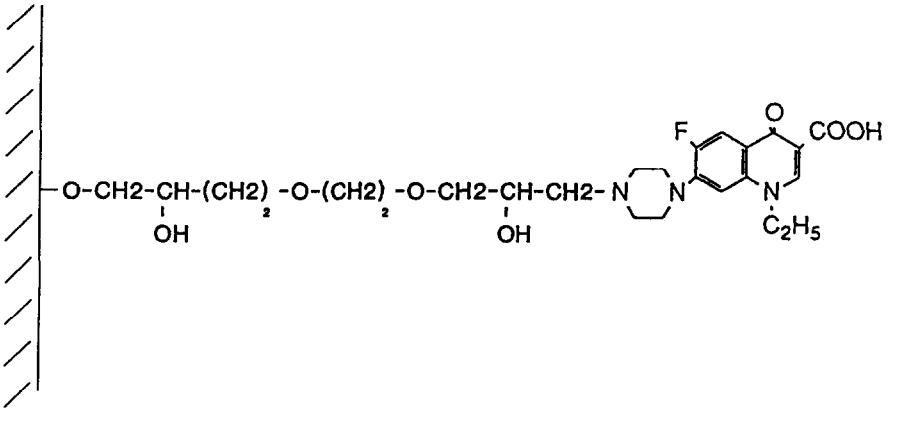

Fig. 1. Structure of norfloxacin immobilized on epoxy-activated Sepharose.

Table 1
Antibacterial activity of fluoroquinolones, their inhibitory activity against DNA gyrase and their affinity for DNA

Quinolone	MIC (mg l^{-1})	MED (mg l^{-1})	K_A ($\times 10^3$ l mol^{-1})[a]		nK_A ($\times 10^3$ l mol^{-1})[b]	
			Without Mg^{2+}	With Mg^{2+}	Without Mg^{2+}	With Mg^{2+}
Sparfloxacin	0.05	0.2	2.6	41	–	–
Pefloxacin	0.2	1	2.6	54	0.7	3.0
Ciprofloxacin	0.05	0.1	2.4	31	0.5	6.7
RP 65 279	>64	50	2.7	10	–	–
Pefloxacin ethyl ester	>128	>35	2.8	3.5	0.9	0.6
BMY 33315	0.1	1	–	–	0.4	3.5
BMY 42230	0.2	10	–	–	0.4	3.1
BMY 43261	3.5	40	–	–	0.1	2.6
BMY 40062	0.03	0.6	–	–	2.4	6.2
BMY 180820	3.5	10	–	–	1.0	3.2
BMY 180821	0.15	0.12	–	–	1.1	3.4

Antibacterial activity was expressed as MICs, the lowest concentration of the antibiotic that inhibited the growth of the culture. The inhibitory activity of a quinolone against DNA gyrase-catalysed supercoiling is expressed as minimal effective dose (MED; the minimum amount of drug required to cause any inhibition of activity).

[a] Experiments were carried out using a fixed concentration of DNA (57 μM of base) and various concentrations of fluoroquinolones, using partitioning in a 6% (w/w) dextran–4% (w/w) PEG system as described under Experimental. Affinity is given by K_A, where K_A is the intrinsic association constant.

[b] As above, but experiments were carried out using a fixed concentration of quinolone (4 μM) and various concentrations of DNA. Affinity is given as nK_A, where n is the number of sites.

latter was always used, because it was cheaper. The representation of Pearlman and Crepy [23] for the binding of ciprofloxacin with and without Mg^{2+} is given in Fig. 2 as an example of the method with fixed fluoroquinolone concentration, and the Scatchard representation for the binding of sparfloxacin is given in Fig. 3 as an example of the "fixed DNA method". In the absence of Mg^{2+}, for the series with fixed quinolone concentration, all the assayed compounds have roughly the same affinity, with nK_A varying from 400 to 1000 l mol^{-1}, with the exception of BMY 43261 ($nK_A = 100$ l mol^{-1}) and BMY 40062 ($nK_A = 2400$ l mol^{-1}). For the series with fixed DNA concentrations, the K_A values varied from 2400 l mol^{-1} for ciprofloxacin up to 2800 l mol^{-1} for sparfloxacin. In the presence of 5 mM Mg^{2+}, the values of nK_A increased by factors of 3–26 for the compounds of the "fixed quinolone" series. For the compounds assayed with fixed DNA concentration, the binding of sparfloxacin, pefloxacin and ciprofloxacin increased, and a second class of sites appeared, with K_A values of $41 \cdot 10^{-3}$, $54 \cdot 10^{-3}$, $31 \cdot 10^{-3}$ l mol^{-1}, respectively. The affinity constant of RP 65279 increased by a factor of 4. For both series, the binding of norfloxacin methyl ester (not shown) and pefloxacin ethyl ester remained unchanged.

3.2. *Affinity chromatographic results*

The chromatographic behaviour of DNA, gyrase and a mixture of both is shown in Fig. 4. In the presence of magnesium, DNA gyrase had no affinity for the column of immobilized norfloxacin. Under the same conditions, DNA was retained and was eluted by the addition of 0.1 M NaCl in buffer E (Fig. 4A). If the mixture of DNA and gyrase was introduced onto the column, the complex was retained and eluted with 0.2 M NaCl (Fig. 4B). The small peak eluted with 0.1 M NaCl in Fig. 4B is probably due to some DNA bound without gyrase on the column. A control experiment carried out using Sepharose without immobilized norfloxacin showed that DNA and gyrase do not bind the resin alone.

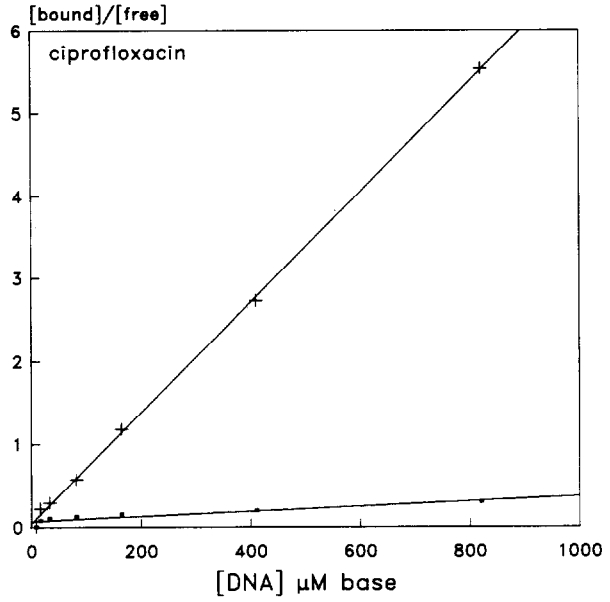

Fig. 2. Binding of ciprofloxacin determined by partitioning, using a fixed concentration of quinolone (4 μM). The ratio of the concentration of bound quinolone to the concentration of free quinolone is plotted against the concentration of DNA, in moles of base per litre. The slope of the curve (regression line) gives nK_A, defined in Table 1. + = Buffer with 5 mM Mg^{2+}; ● = buffer without Mg^{2+}.

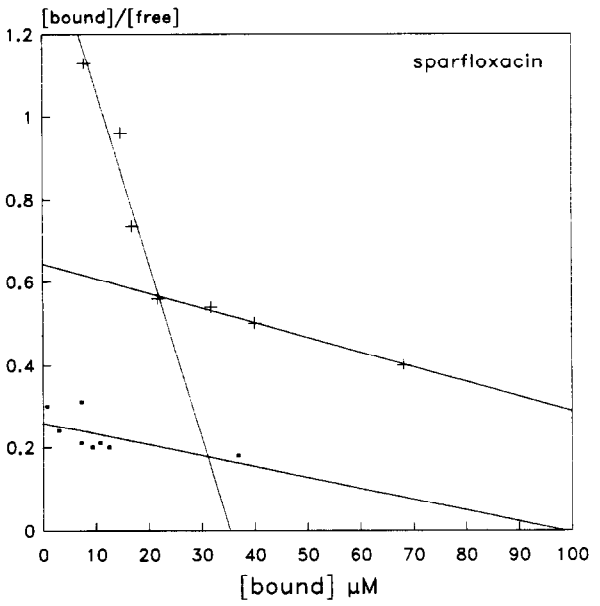

Fig. 3. Determination of K_A, the equilibrium constant of association of sparfloxacin, with calf thymus DNA. The ratio of bound to unbound quinolone is plotted against the molar concentration of bound quinolone. The DNA concentration (57 μmol of base per litre) was kept constant and various amounts of [^{14}C]sparfloxacin were added. Partitioning in the dextran–PEG system was used as described under Experimental. + = Buffer with 5 mM Mg^{2+}; ● = buffer without Mg^{2+}.

4. Discussion

Using partitioning in a PEG–dextran aqueous phase system, it is easily possible to find conditions where DNA totally partitions into the dextra-rich lower phase. Hence it was possible to use such systems to study the interactions between quinolones and DNA. The two-phase partitioning experiments clearly indicated that the binding of quinolone to DNA is mediated by magnesium. In the absence of this ion, the assayed compounds had roughly the same binding parameters, with a mean value of nK_A of 800 l mol^{-1} for the experiments carried out at fixed quinolone concentration, and a mean affinity constant K_A of 2600 l mol^{-1} for the experiments carried out at fixed DNA concentration. We could assume that this binding is totally non-specific, mediated by various factors, such as hydrophobicity, charge and stacking properties of the quinolone. In the presence of Mg^{2+}, the affinity increased for all compounds, except for pefloxacin ethyl ester and norfloxacin methyl ester, the binding constants of which remained unchanged. These latter compounds were unable to bind Mg^{2+} in the same manner as the corresponding acids [13], as this binding needs the presence of both the ketone and the carboxylate. On the other hand, the affinity constant in the presence of Mg^{2+} was not strongly dependent on the activity of the quinolone, because RP 65279, although devoid of activity on both bacteria and gyrase (but able to bind Mg^{2+}), had an affinity constant of 10 000 l mol^{-1} in the presence of magnesium. We conclude that the binding in the presence of Mg^{2+} implies a ternary complex between quinolone, Mg^{2+} and DNA; its formation depends mainly on the ability of the drug to bind Mg^{2+}.

Our results, obtained under equilibrium con-

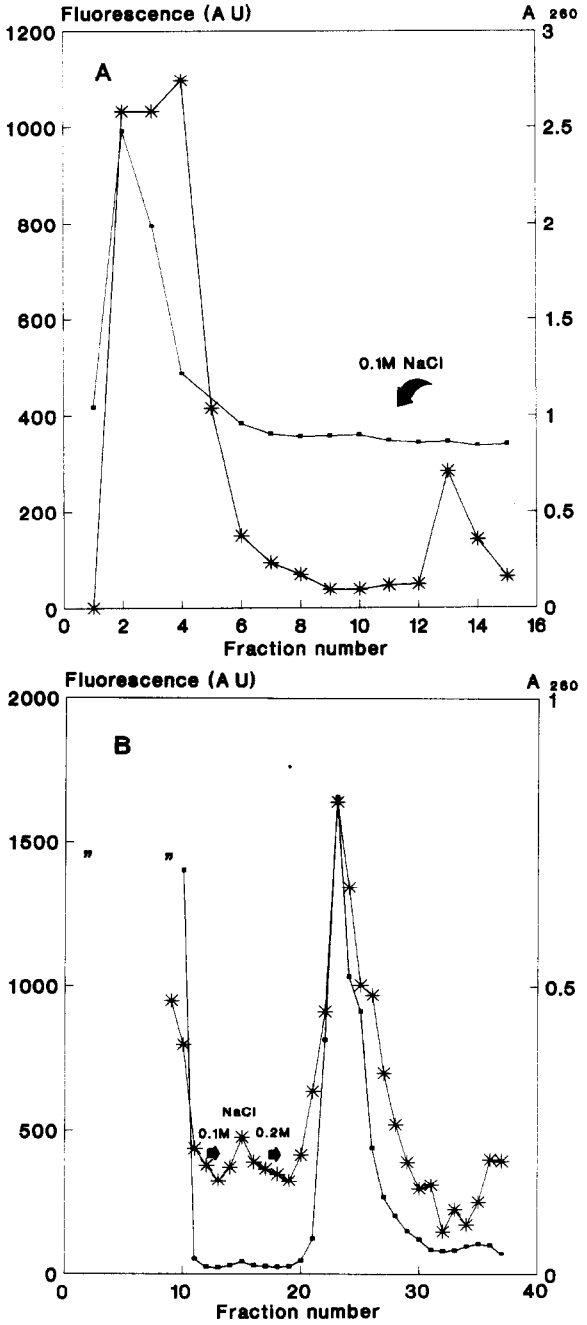

Fig. 4. Chromatography of (A) DNA gyrase or DNA alone and (B) a mixture of both on immobilized norfloxacin (for fraction numbers 0–10, " " indicates that the fluorescence is higher than 2000). The column was equilibrated with the buffer Tris–50 m*M* HCl (pH 7.5)–20 m*M* KCl–2 m*M* $MgCl_2$. Elution was carried out with the same buffer containing 0.1 or 0.2 *M* NaCl as indicated by the arrows. ● = Protein concentration; * = DNA concentration, measured as indicated in Section 2.5. A.U. = arbitrary units.

ditions, can be compared with those of Palù *et al.* [7], who determined the binding constants of [^{14}C]norfloxacin to DNA in the presence of Mg^{2+}, using DNA affinity chromatography and fluorescence experiments. They found a K_A between 1900 and 42 000 l mol^{-1} depending on whether they considered the binding of the quinolone to the DNA–Mg^{2+} complex or of the quinolone–Mg^{2+} complex to the DNA. These values fit with our mean value of 31 000 l mol^{-1}.

The experiments carried out using affinity chromatography on a column of norfloxacin confirmed the results from phase partitioning experiments. Norfloxacin was used as an affinity ligand, because it contained on the piperazine ring an NH function able to react with the epoxide of epoxy-activated Sepharose, and is the desmethyl analogue of pefloxacin. The latter, having an NCH_3 group on the piperazine, could not be immobilized on the resin. The NH function of norfloxacin was the only one able to react with the epoxide, so that the immobilized drug presents the sequence N–CH_2–spacer arm–resin on the piperazine moiety, resembling the N–CH_3 group of pefloxacin (Fig. 1). Therefore, this affinity column should be a good model for pefloxacin. Its use suggested interactions between quinolone, Mg^{2+} and DNA. A relatively low ionic strength (0.1 *M* NaCl) is sufficient to elute the DNA from the column, and this is in good agreement with the low affinity of DNA for fluroquinolone found using two-phase partitioning (K_A = 54 000 l mol^{-1} for pefloxacin). These results completed those of Palù *et al.* [7], who used a DNA cellulose column (*i.e.*, immobilized DNA), and observed binding of norfloxacin on their column only in the presence of Mg^{2+}.

When DNA gyrase was loaded on the column, no appreciable binding was observed, but when the mixture of gyrase plus DNA was loaded on immobilized quinolone, the complex was bound. Moreover, the affinity of the complex was higher than that of DNA alone, as the ionic strength required for elution is slightly higher for the former. This suggests that the quaternary system (DNA–Mg^{2+}–quinolone–gyrase) is more stable than the ternary complex without gyrase.

A possible explanation for the results presented here is that pharmacologically meaningful

recognition between a quinolone and the elements of DNA supercoiling occurs only when DNA, gyrase and Mg^{2+} are simultaneously present. A first step of non-biologically relevant (but Mg^{2+}-dependent) binding would take place between quinolone and DNA, without great differences between various quinolones, provided that they are able to bind Mg^{2+}, as shown here using eleven fluoroquinolones. When gyrase binds to DNA, the presence of the quinolone would then lead the enzyme to adopt slightly different conformations depending on the structure of the quinolone, and this would lead to various impairments of the catalytic activity of the enzyme. This step would then be the pharmacologically relevant one. The proposals made here are in good agreement with the model suggested by Palù *et al.* [7]. Further experiments would be necessary to understand fully the mechanism of inhibition of DNA gyrase by quinolones, and the use of partitioning in dextran–PEG aqueous phase systems offers the great advantage of performing experiments under equilibrium conditions.

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