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A reply: "Do quinolones bind to DNA?"—Yes

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Utilizing equilibrium dialysis and membrane filtration methods, we have shown that quinolones, a class of potent DNA gyrase inhibitors, bind to DNA in both specific and non-specific manners [1]. Specific binding was demonstrated by the cooperative binding of the drug to a saturable site on supercoiled DNA at a concentration close to the K_i (supercoiling inhibition constant against *Escherichia coli* DNA gyrase) of the drug. This specific form of binding is believed to be of biological significance as the apparent dissociation constants (K_d) of a number of selected quinolones, determined by an indirect competition method, were shown to be directly proportional to their inhibitory potencies. The drug binds preferentially (at least one order of magnitude better) to single-stranded than to double-stranded DNA; this leads us to believe that the binding site in the supercoiled DNA is a small denatured bubble which is promoted by negative supercoiling. Recently a preliminary communication by Palu' *et al.* [2] published in this journal questioned the binding of this class of compounds to DNA judged on their inability to detect binding to DNA at a single drug concentration using mainly the fluorescence measurements. Such contradictory observations need clarification. In this communication, I would like to present new experimental evidence, as well as an analysis, which will provide a theoretical basis explaining why the drug does not bind to DNA under their selected experimental conditions. With such analysis it may be concluded that the negative results shown in Fig. 1 of Palu' *et al.* are due to the high cooperativity of the binding and the low ligand concentration used (0.05 μM) which is 1/20 of the K_d value obtained with the binding of norfloxacin to supercoiled ColE1 DNA [1]. The negative results shown in Fig. 2 of their report are due to the improper design of the experiments.

Materials and methods

Materials. Calf thymus DNA was purchased from Worthington Biochemical (Freehold, NJ) and was further purified by phenol extraction. The DNA thermal denaturation procedure was the same as previously described [1]; the denatured DNA gave a 27% increase in absorbance at 260 nm. The final denatured DNA stock solution was dialyzed with the binding buffer [50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), pH 7.4; 20 mM KCl; 5 mM $MgCl_2$; 1 mM dithiothreitol and 1 mM EDTA). [^{3}H]Norfloxacin was the same material used previously [1] but was re-purified by an HPLC procedure [3].

Binding experiments. Procedures for equilibrium dialysis and membrane ultrafiltration binding methods were described previously [1]. Due to the more irregular and thicker Amicon membrane discs manufactured in later batches, the method of data calculation was modified. In this report, the amount of ligand bound to the receptor was calculated by subtracting the free ligand concentration in the reaction mixture from the initial total ligand concentration, both ligand concentrations being determined from the radioactivity counts of the corresponding filtrates with and without the receptors. The quenching of norfloxacin's intrinsic fluorescence upon DNA binding was measured using a Perkin–Elmer model LS-5 fluorescence spectrophotometer. The excitation wavelength used was 340 nm; the absorbance at this wavelength was unaffected by the added DNA.

Results and discussion

It is obvious from our published data (see Fig. 1) that the level of norfloxacin binding to ColE1 or pBR322 DNA drops sharply at low drug concentrations, and that drug binding cannot be detected at 0.05 μM. The molar binding ratio at this low drug concentration may be calculated utilizing Hill's equation (1) which has been employed to fit drug-binding curves [4]:

$$R = \frac{R_m}{1 + (K_d/D)^n} \quad (1)$$

where R, R_m, K_d and D are molar binding ratio, maximum molar binding ratio, apparent dissociation constant, and free drug concentration, respectively, and n is the Hill constant. When $n = 1$, the binding is purely non-cooperative and as n increases the binding is increasingly cooperative.

The K_d value for norfloxacin binding to the primary saturation site is 10^{-6} M when using supercoiled ColE1 DNA [1]. Curve fitting using a non-linear least-squares computer program, RS/1 (Bolt, Beranek & Newman Software Products Corp., Cambridge, MA), revealed that the curve is best fitted with n equal to 4, indicating highly cooperative binding [4]. Since R_m is equal to 5 for ColE1 DNA (Fig. 1A), therefore $R = 3.1 \times 10^{-5}$ drug/DNA which is a number too small to be measured with any workable DNA receptor concentration. The highest concentration of pUC8 molecules used by these authors was 18.7 nM (50 nM × 2000 (P/D)* ÷ 5356 nucleotides per pUC8 DNA). This will result in a reduction of only 0.58 pM or 0.0012% from the initial drug concentration of 0.05 μM.

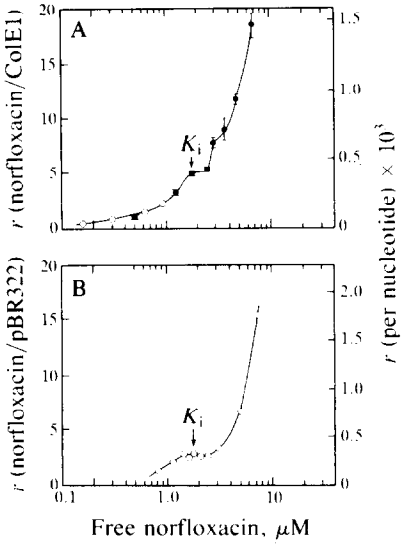

Fig. 1. Binding of norfloxacin to plasmid DNA. Binding mixtures contained 4.7 pmol of ColE1 (A) or pBR322 (B) DNA and the indicated amounts of [^{3}H]norfloxacin. Vertical bars represent standard deviations. Different symbols indicate results obtained from different experiments. Supercoiling K_i values (against *E. coli* DNA gyrase) are shown for comparison. (Reprinted from Ref. 1, Fig. 2.)

* P/D stands for receptor/drug concentration ratio used in Ref. 2. They are distinct from the P and D defined in this paper.

The important concept which we emphasize here is that using a higher receptor:drug ratio (or [P/D]) does not necessarily guarantee a greater binding signal. Working at a lower drug concentration (while holding receptor concentration constant), in general, will give a higher percentage of bound ligand than that at a higher drug concentration, but this is significant only if the binding is non-cooperative ($n = 1$). The following analysis, with the use of some newly obtained binding data, renders further explanation on this.

In a separate publication [4], we studied the binding of unlabeled norfloxacin to supercoiled ColE1 DNA using fluorescence measurements as the method of determining the concentration of the ligand. A binding curve similar to that shown in Fig. 1A was obtained. Computer fitting of the binding data at the primary saturable site gave similar parameters as those obtained previously: $K_d = 0.925 \times 10^{-6}$ M with $R_m = 4.5$ drug per DNA. These binding data are employed here to further explain the change in binding signal as a function of drug concentration and binding cooperativity. Figure 2 shows the original experimental data and two theoretical drug-binding curves drawn according to equation (1) with the newly obtained K_d value (0.925×10^{-6}), and with $n = 1$ or 4. It is seen that the experimental data points fit better with the curve with $n = 4$. When $D \ll K_d$, equation (1) may be simplified to:

$$\frac{R}{R_m} = \left(\frac{D}{K_d}\right)^n$$

Therefore, (R/R_m) decreases as a function of the fourth power of D in the cooperative binding mode, but decreases with the first power of D in the non-cooperative mode. This phenomenon is clearly demonstrated in Fig. 2 by the much sharper sigmoid drop of the (R/R_m) value at the low concentration end in the cooperative binding mode. In theory, a maximum of 2-fold improvement in binding signal may be achieved when the drug concentration is lowered from K_d (the mid-point of the saturation curve) in the non-cooperative binding mode. But in the cooperative binding mode, lowering drug concentration from K_d will adversely result in a drastic reduction of the binding signal (see Appendix).

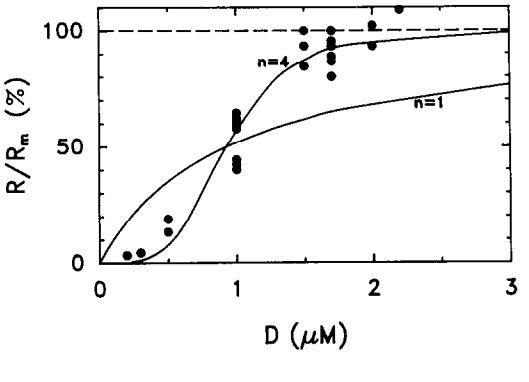

Fig. 2. Binding of norfloxacin to supercoiled ColE1 DNA. The two continued curves are theoretical binding curves drawn according to equation (1) with $K_d = 0.925 \times 10^{-6}$ M and n values as indicated. Experimental data were retrieved from Fig. 6 of Ref. 4 and plotted by assuming an R_m value of 4.5. Experimental conditions were the same as in Fig. 1.

Next, the negative results shown in Fig. 2 of Palu' *et al.* [2] are examined. The authors attempted to use fluorescence measurements to detect the drug binding to DNA by measuring the difference in ligand distribution between two equilibrium dialysis cells. In this experiment, higher drug concentration (0.4 μM) and single-stranded DNA were used ([P] = 2 mM and P/D = 5000; therefore, norfloxacin concentration was equal to 2 mM/5000 = 0.4 μM). This drug concentration is about 1/2 of K_d. The use of single-stranded DNA was expected to facilitate the binding. Unfortunately, the reported experiment is incomplete. Fluorescence measurements cannot be used to determine the concentration of bound ligand in dialysis cells unless one can demonstrate that the fluorescence of the bound drug was not quenched. As shown in Fig. 3A, we found that the intrinsic fluorescence intensity of the drug was quenched when single-stranded DNA was added. The extent of quenching was proportional to the amount of drug bound to DNA (Fig. 3B), indicating a near complete fluorescence quenching of the bound drug. Therefore, the similarity in fluorescence intensities of the drug in the two dialysis compartments shown by these authors simply reflects the equality of free ligand concentrations at equilibrium with the DNA-bound ligand hidden in one compartment "in silence". As a matter of fact, a measurement

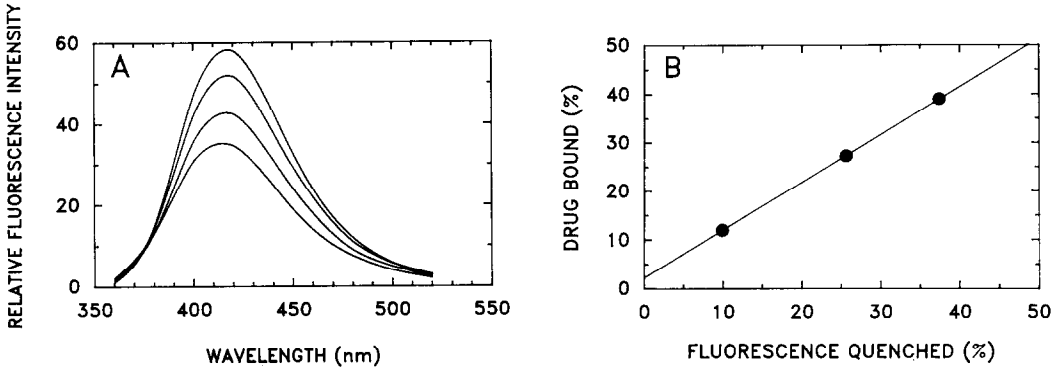

Fig. 3. Quenching effect of DNA on the intrinsic fluorescence of norfloxacin. (A) Emission spectra of norfloxacin (1.2 μM) excited at 340 nm. Curves from top to bottom correspond to the spectra of norfloxacin (1.5 ml) added with 0, 10, 30, and 60 μl of thermally denatured calf thymus DNA (3.65 mg/ml) respectively. The addition of DNA caused no more than 4% of volume change. (B) Correlation of the amount of drug bound and the percent of fluorescence quenching. The percent of drug bound under the same experimental conditions as in (A) was determined by an ultrafiltration technique using [^{3}H]norfloxacin (see Materials and Methods). Both parameters shown have been corrected by the dilution factors.

of fluorescence quenching such as that shown in Fig. 3A is sufficient to indicate the existence of quinolone–DNA interaction. We have used equilibrium dialysis to demonstrate the binding of [^{3}H]norfloxacin to DNA, based on the observation that the radioactivity of the ligand was not altered in the presence of DNA. Significant binding, comparable to that determined by an alternative ultrafiltration method, was observed [1, 4].

It should be mentioned that there is another conflicting report by Le Goffic [5] who attempted to investigate the binding of pefloxacin to DNA gyrase and to DNA using fluorescence and NMR methods. The report concluded that the drug did not bind to DNA, but only to the enzyme. No experimental condition was specified; therefore, analysis of such results was not possible. Binding experiments of quinolones to DNA have become routine in our laboratories. We have obtained crucial information on the specificity and cooperativity of the binding to DNA [4], and the role of DNA gyrase in drug binding to relaxed DNA substrate has also been investigated [3]. Though quinolones bind to DNA, they do not intercalate into DNA [1, 6]. A recent report by Tornaletti and Pedrini [7] confirmed the non-intercalative nature of quinolone binding to DNA by demonstrating the magnesium-dependent DNA unwinding effect of quinolones. The unwinding effect may be required for the specific form of drug binding during DNA gyrase inhibition, as the unwinding effect of a number of quinolones was found to be proportional to their inhibitory potencies. Such DNA unwinding may provide an effect to promote the formation of the gyrase-induced single-stranded DNA pocket as proposed [8].

In summary, sufficient evidence has been presented showing that quinolones bind to DNA. Negative results recently reported in the literature [2] showing an absence of drug binding can be explained on a theoretical basis considering their experimental conditions and by the new experimental information reported herein. The analyses depicted in this report re-emphasize the basic and indispensable concept, i.e. ligand–receptor binding must be investigated at multiple drug concentrations, covering at least the range of biological significance. Without a knowledge of the binding constant and binding cooperativity, one can be misled when interpreting data obtained from a single-point measurement, even though the experiment is performed with a high receptor/drug ratio.

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APPENDIX

The fraction of drug bound to DNA (the binding signal) at total drug concentration D_0 is

$$F = \frac{R}{D_0}[DNA] \tag{2}$$

where $[DNA]$ represents the molar concentration of DNA. Since only a small fraction of total drug binds to DNA, we may assume $D_0 = D$. Substituting equation (1) into (2),

$$F = \frac{R_m}{D[1 + (K_d/D)^n]}[DNA]$$

Let P be the ratio of F_2 to F_1 which are F values at drug concentrations of D_2 and D_1, respectively, then

$$P = \frac{D_1[1 + (K_d/D_1)^n]}{D_2[1 + (K_d/D_2)^n]}$$

If the mid-point of the binding curve is selected as the reference point, i.e. $D_1 = K_d$, then

$$P_k = \frac{2K_d}{D_2[1 + (K_d/D_2)^n]}$$

where P_k is the fractional change in binding signal from K_d to D_2. When $D_2 \ll K_d$,

$$P_k = 2\left(\frac{D_2}{K_d}\right)^{n-1}$$

If $n = 1$ (non-cooperative), $P_k = 2$. Therefore, in theory, a maximum of 2-fold increase in binding signal may be achieved when the drug concentration is lowered from K_d in the non-cooperative binding mode. If the binding is highly cooperative, as in the case of norfloxacin binding to supercoiled DNA ($n = 4$), then

$$P_k = 2\left(\frac{D_2}{K_d}\right)^3$$

In this case, P_k rapidly approaches to 0 when D_2 decreases. Therefore, in the cooperative binding mode, lowering the drug concentration from K_d will adversely reduce the molar binding ratio by an amount far more than what the reduction of drug concentration can contribute to the increase in binding signal.