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# $Mg^{2+}$-Mediated Binding of 6-Substituted Quinolones to DNA: Relevance to Biological Activity

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**Abstract**—The interaction of a number of novel 6-substituted quinolone derivatives with DNA in the presence/absence of magnesium ions has been investigated by fluorometric techniques. The drug-single-stranded nucleic acid interaction is invariantly mediated by the metal ion. In all cases optimal complex formation is found at physiological $Mg^{2+}$ concentration. From titrations at different [$Mg^{2+}$] the binding constant for the ternary drug–DNA–$Mg^{2+}$ complex ($K_T$) has been evaluated. Interestingly, a good relationship is found between $K_T$ and gyrase poisoning activity of the test quinolones ($IC_{50}$), which confirms that DNA-affinity of the quinolone, modulated by $Mg^{2+}$, plays an important role in poisoning the cleavable gyrase–DNA complex and, consequently, in eliciting antibacterial activity in this family of drugs. The results obtained with different 6-substituted compounds supports the idea that position 6 of the drug, besides playing a pharmacokinetic role, is involved in recognition of the enzyme pocket. Our data do not support a mechanism of action based upon quinolone intercalation into B-DNA. 

## Introduction

Quinolones are an exceedingly interesting class of antibacterial drugs.[1] The impressive spectrum of activity and the relatively easy route to their chemical synthesis has recently led to a very large number of compounds available in this family. Structure–activity relationships investigations have shown that some key substitutions at the quinolone ring are required for an active compound.[2,3] Among them, the presence of a fluorine atom at position C-6 appears to be necessary to grant high antimicrobial responses, although its pharmacological role is poorly understood.[4]

In contrast, it was recently shown that an appropriate balance of substitutions, in particular at position C-8, would allow replacement of the fluorine atom, without impairing biological activity.[5–7] Obviously, this fact would open the gate to a new class of quinolones, possibly devoid of undesired side effects, such as phototoxicity, that seem to be related to the presence of the halogenated substituent.[8]

Indeed, it was shown that the biological response is remarkably high when the introduction of an amino group at position C-6 is associated to the presence of a methyl group at position C-8.[9,10] It was further confirmed that, similarly to fluoroquinolones, the new derivatives are able to interfere with the enzyme DNA-gyrase. Classical quinolones poison the catalytic activity of the bacterial gyrase likely by a sterically effective localization in the DNA–enzyme pocket. In fact a key property of this class of compounds rests in the ability of binding to single-stranded DNA.[11,12] Connected to that is the important role played by magnesium ions. In fact, not only quinolones are able to complex this ion, but magnesium is fundamental to make the interaction drug–DNA efficient. Although the detailed structure of this ternary complex is not completely clarified,[13,14] its relevance to the biological activity appears to be proven. In fact, only in the presence of the metal ion, quinolones are able to interact efficiently with gyrase or the gyrase–DNA complex.[15,16]

Key words: Quinolones; magnesium; DNA-binding; single-stranded DNA; DNA-gyrase.
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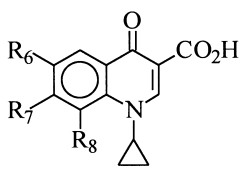
In the present work we decided to investigate in detail the DNA-binding properties of new 6-desfluoro quinolones, bearing either the amino group (**1** and **2**) or a hydrogen (**3**) in the place of fluorine, and to compare them to classical 6-fluoro derivatives (**4** and **5**) in the presence/absence of $Mg^{2+}$ ions. In addition, given the enhancement effects observed in the biological activity tests, a methyl function was introduced at C-8 in all compounds except **5** which, together with ciprofloxacin (**6**, also included in this study), represents the unsubstituted reference compound. Finally, all new tested derivatives bear a *N*-methylpiperazine function at C7, which is eventually replaced by the more hydrophobic 1,2,3,4-tetrahydroisoquinolinyl group in **2** (see Fig. 1). Indeed, this latter substitution proved to be very effective in increasing activity against gram-positive bacteria.[10]

## Results

### Quinolones bind efficiently to magnesium ions

As previously suggested,[13] the affinity of quinolones for magnesium can play a role in the mechanism of drug action. All test compounds showed a dramatic modification of the fluorescence emission quantum yield upon addition of $Mg^{2+}$, characteristic for the formation of a complex with the metal ion.[11,17] In agreement with other quinolones previously investigated, **6** showed an almost twofold increase in fluorometric response in the presence of the metal ion. Similar modifications occur for **3, 4**, and **5** (Fig. 2A). Instead, in the case of aminoquinolones **1** and **2**, a drop in fluorescence emission occurs upon complexation with $Mg^{2+}$ (Fig. 2B). In all cases, saturation of the signal was reached, which allowed us to determine the binding isotherm (Fig. 3). The corresponding binding constants ($K_{Mg}$) assuming a 1:1 complex[11,18] are reported in Table 1. Other possible stoichiometries (1:2 or 2:2) suggested in the literature[17,18] can be safely ruled out in our case due to the very low (sub-micromolar) drug concentrations and the very high ($>10^3$) metal ion:drug ratios employed. In fact, all curve fittings gave substantially better results using the 1:1 rather than any other ratio. Incidentally, the conditions we used are close to physiological. All tested compounds showed $K_{Mg}$ values in the same range as the reference compound and in agreement with values previously obtained for norfloxacin. Only **2** was able to bind $Mg^{2+}$ with a remarkably higher affinity.



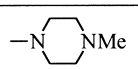
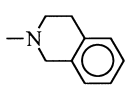
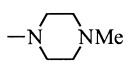
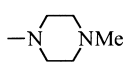
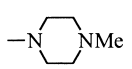
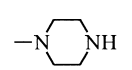

| Compd | $R_6$ | $R_7$ | $R_8$ |
|---|---|---|---|
| **1** | $NH_2$ |  | $CH_3$ |
| **2** | $NH_2$ |  | $CH_3$ |
| **3** | H |  | $CH_3$ |
| **4** | F |  | $CH_3$ |
| **5** | F |  | H |
| **6** | F |  | H |

**Figure 1.** Chemical structure of the test quinolones.

### Quinolone binding to single-stranded (ss) DNA is $Mg^{2+}$-mediated

The binding properties of the test quinolones to ss-DNA were investigated in the presence of different $Mg^{2+}$

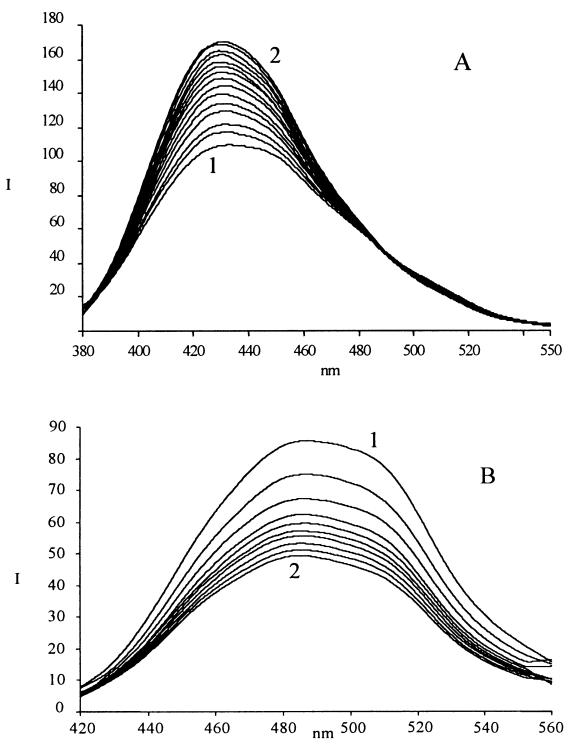



**Figure 2.** Fluorescence emission changes of selected quinolones upon addition of $Mg^{2+}$ in Tris 10 mM, NaCl 20 mM, pH 7.0, 25 °C. Plot A refers to compound **4** ($8.14\times10^{-7}$ M, $\lambda_{exc}=332$ nm), plot B to compound **1** ($2.40\times10^{-6}$ M, $\lambda_{exc}=363$ nm). (1 = free drug; 2 = $Mg^{2+}$-complexed drug.)

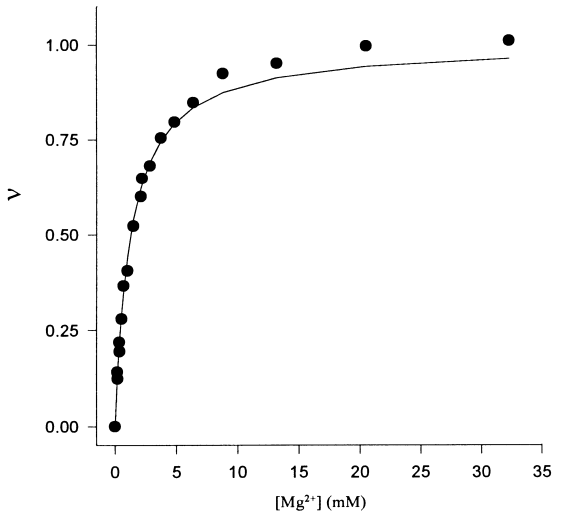



**Figure 3.** Binding isotherm at 25 °C for the **4**–$Mg^{2+}$ complex in Tris 10 mM, NaCl 20 mM, pH 7.0.

**Table 1.** Formation constant for the 1:1 quinolone $Mg^{2+}$ complex at 25 °C and pH 7.0

| Drug | $K_{Mg}$ ($M^{-1}$) |
|---|---|
| **1** | 870 ± 50 |
| **2** | 1620 ± 90 |
| **3** | 530 ± 40 |
| **4** | 800 ± 40 |
| **5** | 530 ± 40 |
| **6** | 720 ± 20 |

concentrations. It is worth to note that in the absence of metal ions in solution none of the drugs showed significant modifications of the spectroscopic properties even upon addition of a large ss-DNA excess. The same behavior was observed in the presence of a $Mg^{2+}$ concentration higher than 20–30 mM. Binding effects were instead detectable at intermediate concentrations of the metal ion (0.5–15 mM), as previously found for norfloxacin.[11] Addition of DNA to the quinolone at a given $Mg^{2+}$ in the above concentration range causes dramatic changes in the fluorescence emission of the quinolone. This is clearly evident in Figure 4, where the emission intensities in the presence/absence of the nucleic acid are plotted as a function of $Mg^{2+}$ concentration. From the fluorescence changes it is possible to obtain the fraction of quinolone bound to DNA and to elaborate the data in terms of Scatchard plot.[17] The binding constants, $K_i$, at different $Mg^{2+}$ concentrations are reported in Table 2. Clearly, the concentration of the metal ion plays a critical role in terms of quinolone affinity for the single-stranded nucleic acid, as practically no affinity is exhibited by any of the drugs for the DNA counterpart in the absence of metal ion or in excess of it. In relative

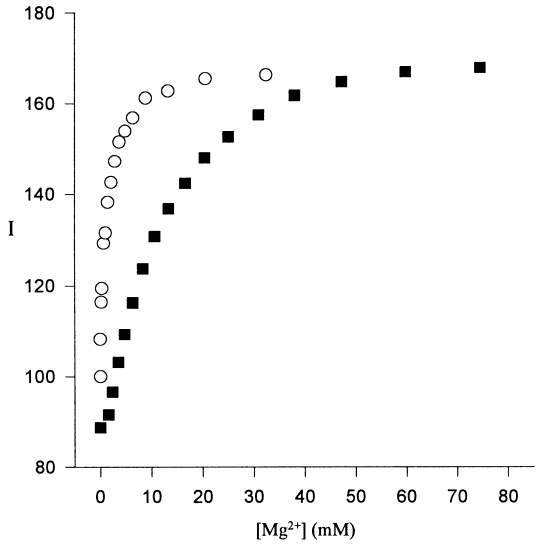



**Figure 4.** Modifications of **4** fluorescence emission as a function of $Mg^{2+}$ concentration in Tris 10 mM, NaCl 20 mM, pH 7.0, 25 °C. ○ in the absence of ss-DNA, ■ in the presence of 1.8 mM (base residue) ss-DNA.

terms, the fluoro derivative **5** exhibits the highest overall affinity for the nucleic acid at each $Mg^{2+}$ concentration tested, followed by the desfluoro derivative **3** and, in a decreasing order of binding efficiency, by **6** and **4**. On the other hand, the amino derivatives **1** and **2** show a remarkably lower $K_i$ value. The changes in affinity for the nucleic acid as a function of $Mg^{2+}$ are described by a bell-shaped curve (Fig. 5), as it was previously found for norfloxacin.[11] Invariantly, the maximum is centered around 1 mM metal ion. This clearly indicates the participation of magnesium in the complex (i.e. the formation of a ternary complex involving drug, ss-DNA, and $Mg^{2+}$).[11]

The relevant equilibria in solution are the following (corresponding binding constants in parentheses), where Q, M and QM were previously defined, $D$ is the nucleic acid, DM is the DNA–$Mg^{2+}$ complex and $T$ is the ternary complex:

$$\begin{aligned} Q + M &\rightleftharpoons QM(K_{Mg}) \\ D + M &\rightleftharpoons DM(K_D) \\ QM + D &\rightleftharpoons T(K_1) \\ Q + DM &\rightleftharpoons T(K_2) \end{aligned}$$

with the overall ternary complex formation:

$$Q + M + D \rightleftharpoons T(K_T)$$

Considering that $K_T = K_{Mg}K_1 = K_D K_2$, and rearranging the equations previously reported using the same set

**Table 2.** Binding constants of the test quinolones to ss-DNA at 25 °C, pH 7.0, and various $Mg^{2+}$ concentrations

| $[Mg^{2+}]$ (mM) | $K_i$ ($M^{-1}$) | | | | |
|---|---|---|---|---|---|
| | **1** | **2** | **3** | **4** | **5** |
| 0 | ≈0 | ≈0 | ≈0 | ≈0 | ≈0 |
| 1 | 2180 ± 120 | 1440 ± 130 | 6860 ± 300 | 6100 ± 200 | 6190 ± 10 |
| 3 | 1480 ± 60 | n.d. | 5400 ± 90 | 4590 ± 10 | 4370 ± 80 |
| 10 | 820 ± 50 | n.d. | 2000 ± 70 | 2180 ± 70 | 1870 ± 30 |
| 25 | ≈0 | n.d. | 375 ± 29 | 295 ± 43 | 380 ± 34 |

n.d. = not determined.

of equilibria,[11] it is possible to calculate the relationship between the quinolone binding constant at a given $Mg^{2+}$ concentration ($K_i$ in Table 2) and the overall binding constant $K_T$.

In fact, we obtain:

$$\frac{K_i}{K_T} = \frac{[M](1-2d)^2}{(1-K_{Mg}[M])(1-d^2)} \quad (1)$$

where

$$d = \frac{1}{2}\left(1 - \frac{1}{\sqrt{1+4K_D[M]}}\right)$$

Therefore, by the best fit of eq (1) as a function of the magnesium concentration (Fig. 5) the value of $K_T$ can

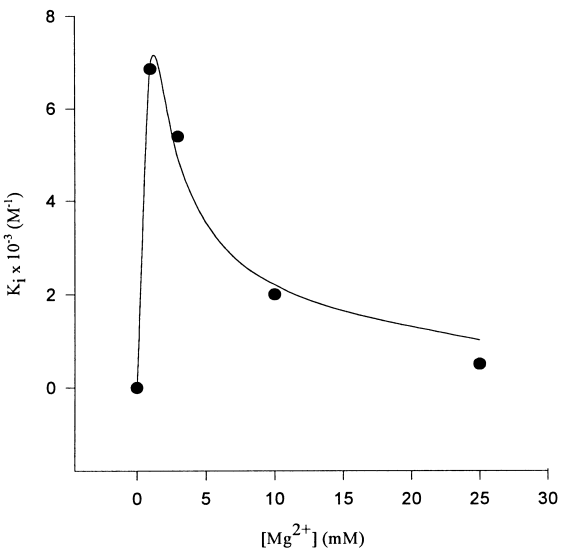



**Figure 5.** Dependence of the ss-DNA binding constant $K_i$ on $Mg^{2+}$ concentration. The full line represents the best fit of eq (1) (see text) for the experimental set of data.

**Table 3.** Ternary complex formation constants ($K_T$) for the test compounds at 25 °C and pH 7.0

| Drug | $K_T \times 10^{-7}$ ($M^{-2}$) |
|---|---|
| **1** | 1.05 ± 0.26 |
| **2** | 1.00 ± 0.50 |
| **3** | 4.35 ± 1.73 |
| **4** | 2.15 ± 0.39 |
| **5** | 5.74 ± 0.73 |
| **6** | 3.62 ± 0.16 |

be obtained as reported in Table 3. The value for **2** is inferred from one $K_i$ value only, due to the rather low binding affinity at high $Mg^{2+}$ concentrations. Hence, it can be affected by considerable error. All tested drugs exhibit $K_T$ values in the $10^7\,M^{-2}$ range, which points to the efficiency of the metal-ion-mediated DNA-binding process. **5** appears to be the most effective in the recognition of ss-DNA, followed by **3, 6**, and **4**. The amino derivatives **1** and **2** show remarkably lower affinity values.

### Quinolone binding to ds-DNA is not significant at physiological conditions

To evaluate whether the observed binding to DNA is peculiar to the single-stranded form, all derivatives were additionally tested for their ability to bind to ds-DNA in the classical B-form. Confirming previous results on norfloxacin,[11] in the presence of 1–25 mm $Mg^{2+}$ none of them showed any significant modification of the fluorometric properties even upon adding a large excess of nucleic acid. In the absence of metal ion, only **6** and **5** showed a modest affinity for B-DNA, which totally vanished upon increasing salt concentration ($Na^+$ or $Mg^{2+}$) to physiological values (Fig. 6). Hence, this type

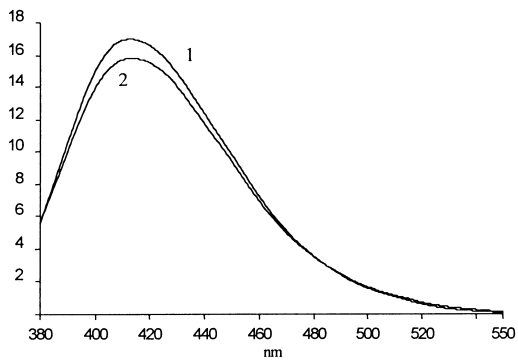



**Figure 6.** Effect of ds-DNA addition on the fluorescence emission properties of **5** ($1.69\times10^{-7}$ M, $\lambda_{exc}$ = 330 nm), at 25 °C in Tris 10 mM, NaCl 150 mM, EDTA 1 mM, pH 7.0. (1 = in the absence of ds-DNA; 2 = in the presence of a large excess (≈2 mM) of ds-DNA.)

of binding is essentially due to electrostatic interactions, most likely involving the C7 basic substituent in the protonated form and the negatively charged polymicleotide backbone.

## Discussion

The results presented here show that the structural modifications introduced in the test quinolones modulate both $Mg^{2+}$ and DNA binding. As it could be expected, all derivatives bind $Mg^{2+}$ efficiently. In fact, each one possesses the carbonyl and carboxyl moieties comprising the coordination site for the metal ion.[13,18] According to the data reported in Table 1, it appears that *N*-methylation of the piperazinyl group at C-7 leads to less effective complexation probably due to a change in the amine p$K_a$ and hence in the protonation equilibria (**6** versus **5**). Methylation at C-8 increases the affinity for the metal ion (**5** versus **4**) by possibly increasing the electron density at the coordination sites. As far as substitution at C-6, fluorine or amino groups favor complex formation to a comparable extent (**4** versus **1**), while the reverse occurs when hydrogen is present (**4** versus **3**). A special case is represented by **2**, as it exhibits the highest $K_{Mg}$ value. This is clearly ascribable to the presence of an uncharged tetrahydroisoquinolinyl group at C-7. In fact, while all other compounds are zwitterionic at neutral pH and do not bear a net charge,[19,20] **2** is in the anionic (carboxylate) form at the same conditions. A negatively charged compound will likely bind to the metal ion more efficiently than the globally neutral congeners. Comparing the $K_{Mg}$ data with the $IC_{50}$ values found for the inhibition of DNA-gyrase for the test drugs,[10] a poor correlation can be found (Fig. 7A). Indeed, even not including **3**, which lies clearly out of the expected range, a linear regression analysis gives unsatisfactory $r^2$ values of about 0.3. Thus, magnesium binding to the drug does not appear to represent per se a key parameter to unveil structure–activity relationships.

On the other hand, the presence of an appropriate concentration of $Mg^{2+}$ is a fundamental requirement to confer a significant affinity for ss-DNA to all test drugs (and the other classical quinolones so far tested).[13,15] Considering the physiological presence of magnesium ions in the cell nucleus, this points to the biological relevance of a ternary quinolone–DNA–$Mg^{2+}$ complex. In addition, independently of the structural modifications introduced, optimal complex formation is always occurring around 1 mm [$Mg^{2+}$], very close to the conditions found in bacterial cells.[11]

The ternary complex formation constants, $K_T$, obtained from eq (1), take into an account both the effects of the

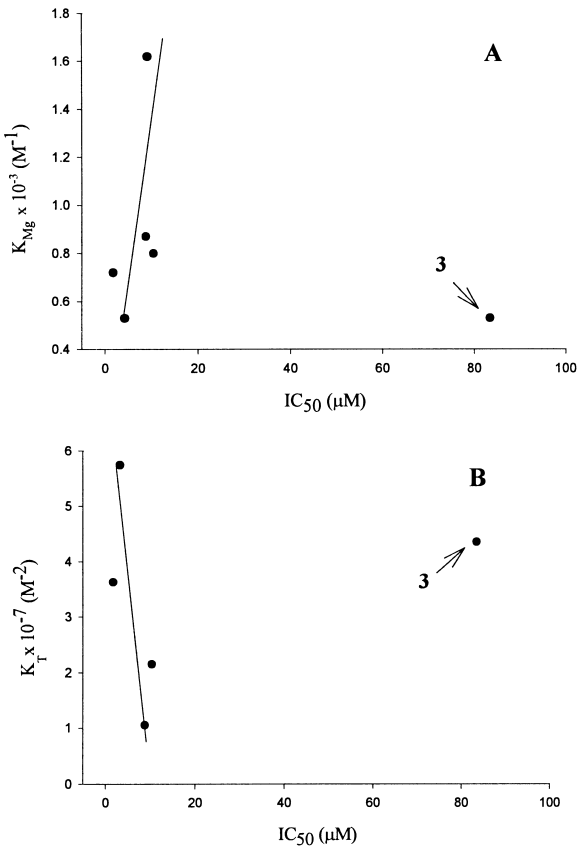



**Figure 7.** Plot of *E. coli* DNA-gyrase 50% inhibitory concentration ($IC_{50}$)$^{vs}$. $K_{Mg}$ (plot A) or $K_T$ (plot B) for the test quinolones.

quinolone per se and the influence of $Mg^{2+}$ on the ss-DNA binding properties (Table 3). The C-6 fluorinated compounds exhibit high binding affinity, **5** being most effective. In this series, methylation at C-8 slightly disfavors complexation, whereas the reverse is true for N-methylation at the C-7 pyperazinyl residue. The latter fact is possibly ascribable to small changes in protonation equilibria (a tertiary amine is generally more basic than a secondary amine) as suggested above when discussing the metal ion complexation properties. Amine substitution at position 6 disfavors the ternary complexation process (see $K_T$ data for **1** and **2**).

On the contrary, and interestingly, the C-6 unsubstituted derivative **3** shows the highest propensity for ss-DNA binding among the C-8 methylated quinolones we have studied. Since both F- and $NH_2$- residues tend to increase the electron density on the quinolone moiety, this might represent the reason for a lesser interaction of this portion of the drug with the electron-rich nucleic acid backbone. In any event, it is worth emphasizing that the $Mg^{2+}$-mediated binding for ss-DNA remains effective for all tested compounds.

To probe the pharmacological significance of the quinolone–DNA–$Mg^{2+}$ tertiary complex it is useful to plot the affinity constant $K_T$ (Table 3) as a function of the DNA-gyrase inhibition data.[10] The results are presented in Figure 7B. With the notable exception of **3**, all piperazinyl quinolones show a satisfactory correlation between $K_T$ and $IC_{50}$ values ($r^2$ of the plot = 0.85). This confirms that (magnesium-mediated) ss-DNA-affinity of the quinolone plays an important role in affecting gyrase inhibition by this family of drugs. Supporting a mechanism of action originally proposed by us and further elaborated by other groups,[11–13] this is also suggestive that poisoning of the cleavable complex, which is normally caused by the formation of a ternary drug–DNA–enzyme adduct for other known inhibitors, would be originated by a quaternary system, including $Mg^{2+}$ in the case of quinolones.

The finding that **3** is an outlier deserves some further comment. In fact, substitution of F with $NH_2$ at C-6 preserves gyrase inhibition efficiency of the new compounds.[10] On the contrary, when H is present at the same position enzyme poisoning occurs at exceedingly high concentrations of the quinolone. This fact points to specific (perhaps hydrogen bonding) contacts between the drug and DNA-gyrase at C-6. Clearly, lack of this type of interactions when using **3** renders recognition of the cleavable complex by the quinolone remarkably less effective even in the presence of an elevated affinity for DNA. Therefore, besides affecting pharmacokinetic properties such as bioavailability and cell penetration,[1] the introduction of a fluorine atom at C-6 appears to play a pharmacodynamic role too. Although other 6-H quinolones should be examined to fully confirm, this conclusion, our data agree with a recent SAR study suggesting an interaction between the fluorine at position 6 and the quinolone resistance determining region of the gyrase.[4]

A final property that the new derivatives share with clinically relevant quinolones, such as norfloxacin or ciprofloxacin, is their keen preference for ss-DNA as compared to ds-DNA. Experimental evidence was recently presented that quinobenzoxazines, a class of cytotoxic compounds structurally related to quinolones, are able to intercalate into the DNA double helix in the presence of $Mg^{2+}$.[12] From these data, the above-mentioned SAR study proposes that one of the important parameters to describe the quinolone mode of action is represented by drug intercalation into double-stranded DNA in the presence of magnesium.[4] However, quinobenzoxazines exhibit a much more extended planar ring system than quinolones do. Hence, the former will exhibit substantially more efficient stacking interactions with DNA base-pairs when intercalated, and the energy gained in the stacking process will compare favorably with the energy spent in unwinding DNA to allow intercalation. Conversely, classical antibacterial quinolones, producing poorer stacking, will need to be 'helped' for their binding to the nucleic acid and require a local disruption (unwinding) of the double helix to be able to interact. Hence, only a remarkably distorted (unwound) form of B-DNA appears to represent a suitable binding site for the quinolone family. We have already shown that, unlike linear double-stranded DNA, covalently closed circular DNA does indeed possess a small number of quinolone binding regions in the presence of physiological $Mg^{2+}$ concentrations, which presumably correspond to unwound nucleotide sequences.[11] Hence, in general agreement with the mechanistic information thus far available, the biologically relevant DNA target must possess a single-stranded (or a single-stranded-like) structure, rather than a double helical conformation, even in the presence of $Mg^{2+}$. Clearly, a gyrase-unwound duplex DNA would fulfill the above requirements.

## Conclusions

Our data confirm the importance of $Mg^{2+}$-mediated interactions in the mechanism of action of antibacterial quinolones. Indeed, the metal ion is required for effective DNA-binding, and the equilibrium constant for ternary DNA–drug–$Mg^{2+}$ complex formation is well related to quinolone ability to inhibit DNA-gyrase. These findings should be considered in the future design of novel DNA-gyrase-directed chemotherapeutic agents.

## Experimental

### Drugs and DNAs

The tested quinolones were prepared as previously described.[9,10] Stock solutions (1 mg/mL) were made in DMSO and diluted to the working concentration in the desired buffer. Ciprofloxacin was a kind gift of Glaxo Wellcome Ltd. (S.p.A.). Stock solutions were made in water and diluted to the working concentration in the desired buffer. Double-stranded calf thymus DNA (ds-DNA) was from Sigma Chemical Company and was used without further purification. Single-stranded calf lung DNA (ss-DNA) of average molecular weight 30,000 was kindly provided by Crinos S.p.A., Como, Italy. DNA stock solutions were made in the desired buffer and concentrations were evaluated by measuring the absorbance at 260 nm using a molar extinction coefficient of 6600 $M^{-1}cm^{-1}$ for ds-DNA and of 8300 $M^{-1}cm^{-1}$ for ss-DNA.

### Magnesium binding

The binding process between the tested quinolones and $Mg^{2+}$ ions was investigated by fluorometric titrations performed on a Perkin–Elmer LS 50 instrument. The fluorescence intensity of a quinolone solution in Tris 10 mM, NaCl 20 mM, pH 7.0, 25 °C was measured following addition of known (small) amounts of a 0.3 M solution of $Mg(ClO_4)_2$ in the same buffer. Each solution was filtered through a 0.45 μ filter before use to eliminate any particulate material that would interfere with the fluorescence response. Due to the large excesses of $Mg^{2+}$ (millimolar range) compared to the quinolone (micromolar range) only the presence of the 1:1 equilibrium was taken into account. The related association constant $K_{Mg}$ for this equilibrium is defined by the equation:

$$K_{Mg} = \frac{[QM]}{[Q][M]}$$

where Q is the quinolone, M is the magnesium ion and QM represents the complex between quinolone and magnesium. Considering that at any $Mg^{2+}$ concentration the observed fluorescence emission signal F is:

$$FC_t = F_o[Q] + F_\infty[QM]$$

where $F_o$ is the fluorescence of the quinolone in the absence of magnesium, $F_\infty$ its fluorescence when bound to magnesium ions, and $C_t$ is the total quinolone concentration, defined as:

$$C_t = [Q] + [QM]$$

At every $[Mg^{2+}]$ we can therefore calculate the fraction of binary complex quinolone-magnesium $\nu$, defined as [QM]/Ct:

$$\nu = \frac{F - F_o}{F_\infty - F_o}$$

Assuming that [M] is essentially the total magnesium concentration (the amount of complex formed is micromolar or less, whereas the total metal ion is in the millimolar range), it was then possible to calculate the association constants $K_{Mg}$ for the binary complex formation from a best fitting of the curve $\nu$ versus $[Mg^{2+}]$.

### DNA binding

Fluorometric titrations at constant quinolone concentration and ionic strength were performed by adding known amounts of DNA in the presence or absence of fixed concentrations of $Mg^{2+}$. The excitation wavelengths used were above 300 nm to avoid quenching of the quinolone fluorescence signal due to DNA absorption. In addition, calibration curves in the absence of drug were built to take into an account any minor contribution arising from solvent and/or DNA. As previously described, the amount of free ($C_f$) and bound ($C_b$) drug were directly calculated from the fluorescence emission intensity at fixed wavelength. The binding constant $K_i$ was then calculated using the McGhee and Von Hippel neighbor exclusion model.[21] The exclusion parameter with ss-DNA was always close to 2.

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