Complexes between fluoroquinolones and calf thymus DNA: binding mode and photochemical reactivity

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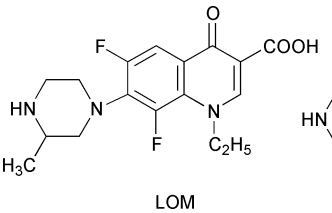
The binding of the highly photocarcinogenic and phototoxic fluoroquinolones (FQs) lomefloxacin (LOM) and enoxacin (ENX) to calf thymus DNA (ct-DNA), as well as the photoreactivity of the FQs-ct-DNA complexes, have been investigated at neutral pH through the combination of several spectroscopic techniques. Both the nature and efficiency of the binding are markedly dependent on the buffer concentration. In 10^{-3} M phosphate buffer the interaction with ct-DNA seems to involve mainly the cationic form of both FQs, which bind to the helix with different efficiency. The overall results are consistent with a binding mode characterized by a close proximity of the chromophores to the DNA bases. An increase of one order of magnitude of the buffer concentration induces a displacement of the molecules from the DNA interior, leading to significant decrease of the association constants. The formation of external complexes involving mainly the zwitterionic forms and characterized by similar binding efficiency for both FQs appears highly probable under these experimental conditions. In this overall scenario, the positively charged piperazinyl ring of both molecules is thought to act as a pivot, going from one binding mode to another. The FQs-DNA complexes reveal a remarkably different photoreactivity. The LOM-ct-DNA complex is characterized by a photodefluorination yield similar to that observed for the free molecule whereas the ENX-ct-DNA complex is basically unphotoreactive. A rationale for these effects is provided. The relation of the overall results to the adverse side reaction photoinduced by these drugs is also commented upon briefly.

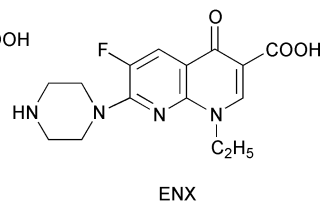
Fluoroquinolones (FQs) are a class of compounds widely used as broad-spectrum antimicrobial agents.[1] They develop their pharmacological action *via* specific inhibition of sub-unit A of the bacterial gyrase, an enzyme that controls DNA shape.[2] In the last few years these molecules have received growing attention from interdisciplinary areas of the scientific community due to both practical purposes and fundamental aspects. Actually, despite the important step forward in infective therapy represented by FQs, a serious drawback for their use is their phototoxic and photocarcinogenic activity.[3,4] Beyond these concerns, from a strictly photochemical point of view, the interest of photochemists in this class of molecules has been captured by the fact the many of them undergo photodefluorination,[5] a very uncommon process in the wide arena of fluoroaromatic photochemistry, due to the strength of the C–F bond (dissociation energy *ca.* 523 kJ mol^{-1}). In this regard, only rare examples have been reported.[6–8] The photodefluorination process was suggested to have a decisive role in the entire photosensitization process involving biological substrates.[3,5,9,10] However, only in recent years have detailed studies addressing the characterization of both stable and transient species involved in the photodegradation of some of these derivatives appeared in the literature.[11–19]

As stated above, FQs inhibit the bacterial gyrase. Although the exact mechanism of this action is still unclear, there is evidence that FQs interact directly with DNA in synergy with the gyrase enzyme.[20–22] Therefore, DNA is thought to be one of the main cellular targets responsible for the aforementioned drug-photoinduced disorders.

The picture emerging from these considerations suggests that in order to gain a deeper and more appropriate understanding of the molecular basis of such adverse reactions photoinduced by FQs, it is of fundamental importance to elucidate the binding features and photochemical behavior of complexes between FQs and DNA. Nevertheless, only a limited number of contributions addressed to this topic have been reported up to now.[23] Binding efficiency, association mode and photoreactivity of these complexes represent key factors that can play dominant roles in determining both the amount and mechanism of the observed noxious side-photoeffects. With this in mind we have focused our attention on the binding properties of lomefloxacin (LOM) and enoxacin (ENX) to calf thymus DNA (ct-DNA), as well as to the photoreactivity of these FQ-DNA complexes at neutral pH.



LOM



ENX

LOM and ENX are two FQs well known for being able to induce remarkable photocarcinogenic and phototoxic reactions[3,4,9,24] and are characterized by efficient photodefluorination pathways.[11–15] LOM is not only much more phototoxic, photomutagenic and photocarcinogenic than other FQs[3,4,25,26] but also one of the most photodegradable.[11–14] Although photodefluorination is the sole photoreaction occurring for these two compounds,[11] LOM and ENX are characterized by different photodefluorination mechanisms. In fact, the loss of fluoride is mediated by a singlet state in the case of LOM whereas a triplet photoreactivity has been ascertained for

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ENX.[13,15] Furthermore, recent steady-state and time-resolved spectroscopic investigations have pointed out an unusually active role of the phosphate buffer in modulating both the efficiency and nature of FQ photodecomposition through static and dynamic quenching processes of the lowest excited states.[12,19] This unexpected role of the inorganic salt spurred us to perform this work at two different phosphate buffer concentrations in order to better correlate DNA affinity and photoreactivity for both FQs.

Results

Absorption spectra

The absorption spectra of LOM and ENX recorded in the presence of increasing amounts of ct-DNA in 10^{-3} M phosphate buffer at pH 7.4 are shown in Fig. 1.† Significant hypochromic effects, accompanied by change in the shape of the absorption bands, and fairly clear isosbestic points were observed in both cases. These findings provide a first indication of the formation of ground-state complexes of LOM and ENX with double-helical ct-DNA. Moreover, the presence of the isosbestic points suggests homogeneity of the FQs-ct-DNA binding. In order to obtain the binding constants, K_b, related to the two complexes we used the half-reciprocal plot of the absorption titration data according to eqn. (1):[27]

$$[\text{ct-DNA}]_{bp}/(\varepsilon_A - \varepsilon_F) = [\text{ct-DNA}]_{bp}/(\varepsilon_B - \varepsilon_F) + 1/K_b(\varepsilon_B - \varepsilon_F) \quad (1)$$

Here ε_A, ε_F and ε_B correspond to $A_{obs}/[FQ]$, the extinction coefficient for the free FQ, and the extinction coefficient for the FQ complex in the totally bound form, respectively.[27] By the ratio of the slope to intercept of the plots reported in the insets of Fig. 1, K_b values of $1 \times 10^3 \pm 80$ and $2.7 \times 10^3 \pm 200$ M^{-1} were found for LOM and ENX, respectively.

Increase of the buffer concentration by one order of magnitude (10^{-2} M) led to less pronounced spectral changes upon addition of ct-DNA (data not shown).‡ The treatment of these data using eqn. (1) gave K_b values significantly smaller than those obtained at lower buffer concentration but basically the same for both compounds at $4 \times 10^2 \pm 50$ M^{-1}.

Induced circular dichroism

Further evidence for the interaction between the investigated FQs and ct-DNA was provided by induced circular dichroism (ICD). Due to the absence of chiral centers the compounds are not optically active by themselves in aqueous solution. As shown in Fig. 2 addition of ct-DNA to solutions of LOM and ENX in 10^{-3} M phosphate buffer at pH 7.4 induces optical activity as a consequence of the close proximity of FQs with the asymmetric environment of the biopolymer helix. It is noteworthy that although the negative ICD bands are quite broad, their absorption maxima correspond fairly well to those of the absorption bands, thus ruling out the existence of exciton splitting phenomena. No ICD signals were observed when the measurements were made in 10^{-2} M phosphate buffer, even at DNA concentrations higher than 10^{-3} M, for which a significant amount of complex is formed.

Fluorescence studies

The interaction of FQs and DNA was further confirmed by fluorescence measurements. The fluorescence spectra were

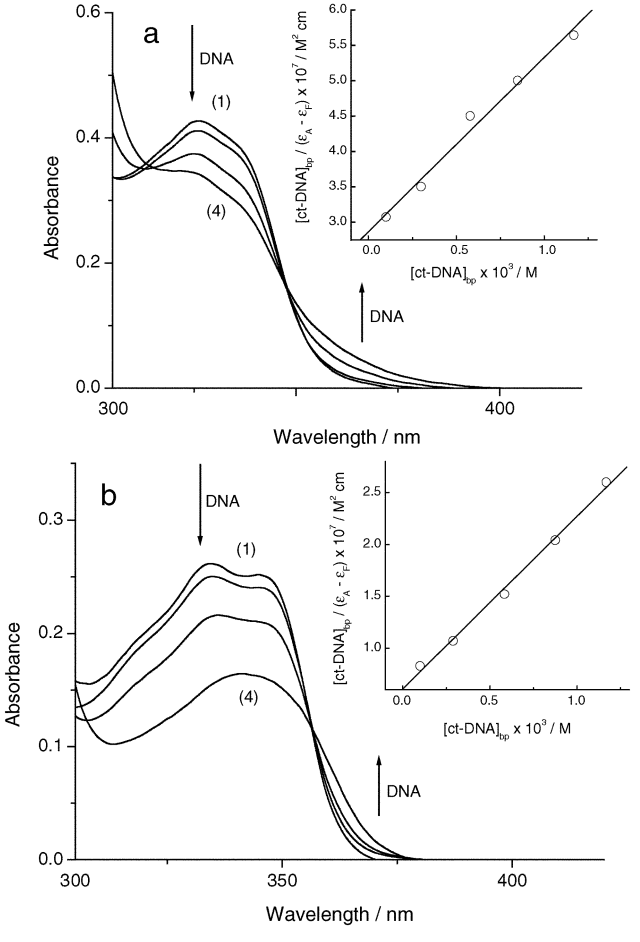


Fig. 1 Absorption spectra of (a) LOM (4.5×10^{-5} M) and (b) ENX (1.6×10^{-5} M) in 10^{-3} M phosphate buffer, at pH 7.4, in the presence of increasing amounts of ct-DNA in the range 0–1.2×10^{-3} M (some spectra are omitted for the sake of clarity). Cell path: 1 cm. The insets show the half-reciprocal plots of FQs binding with ct-DNA determined at 325 nm for LOM and 334 nm for ENX.

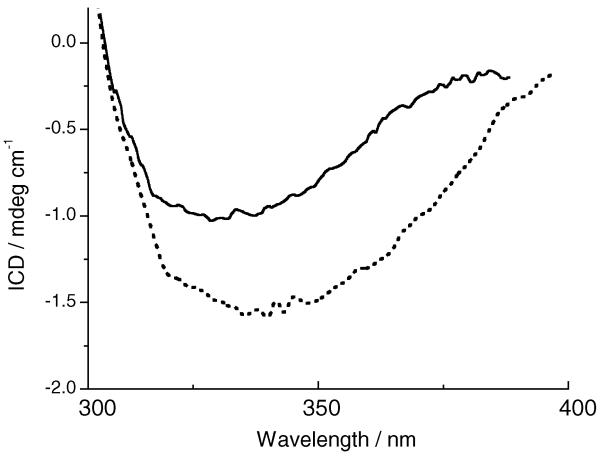


Fig. 2 Induced circular dichroism of the (——) LOM-ct-DNA and (- - -) ENX-ct-DNA complexes in 10^{-3} M phosphate buffer solution, pH 7.4. $[\text{ct-DNA}]_{bp} = 10^{-3}$ M; $[\text{drug}] = 3.3 \times 10^{-5}$ M. Cell path: 1 cm.

† ct-DNA does not absorb in the wavelength windows observed.

‡ Due to the NaCl contained in the DNA used, the actual values of the ionic strength in the two sets of experiments were *ca.* 4×10^{-3} and 1.4×10^{-2}, respectively. These values were estimated by a calibration of K_b as a function of buffer concentration after desalting DNA.

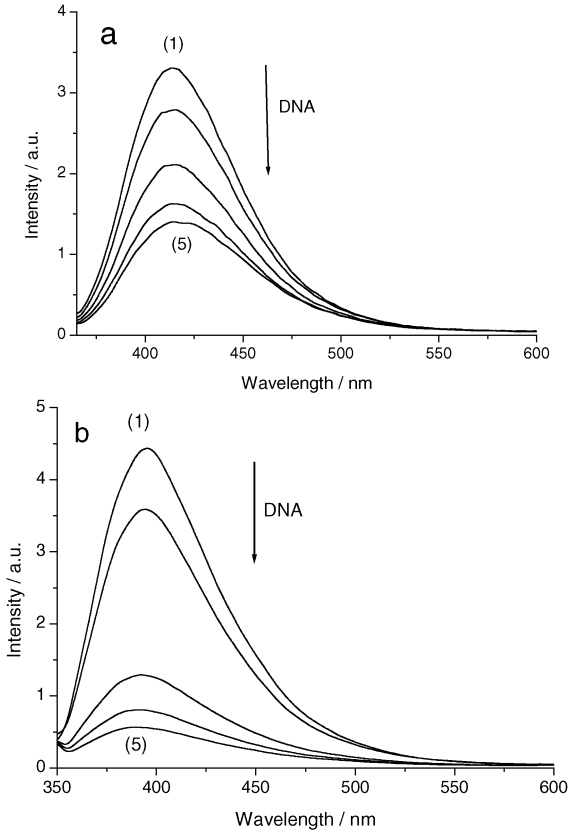


Fig. 3 Fluorescence emission spectra of (a) LOM (4.5×10^{-5} M) and (b) ENX (1.6×10^{-5} M) with increasing concentration of ct-DNA in the range 0–1.2×10^{-3} M and recorded in 10^{-3} M phosphate buffer solution, pH 7.4. $\lambda_{exc} = 348$ and 356 nm (isosbestic points) for LOM and ENX, respectively.

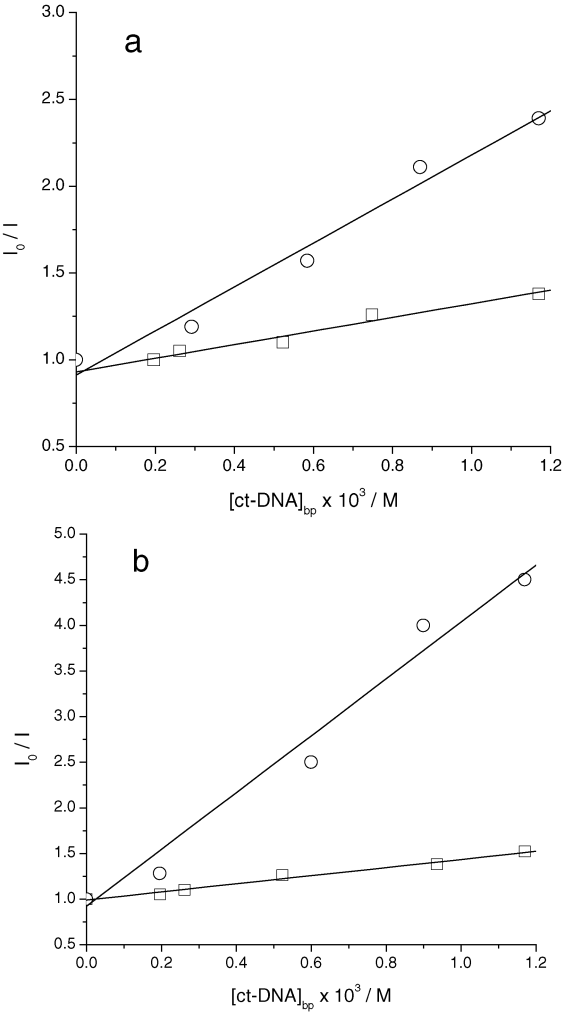


Fig. 4 Stern–Volmer plots related to the fluorescence quenching of (a) LOM and (b) ENX by ct-DNA and obtained in (○) 10^{-3} M and (□) 10^{-2} M phosphate buffer at pH 7.4.

recorded using excitation at the isosbestic points in order to circumvent the trivial changes in fluorescence associated with variations in absorbances. As displayed in Fig. 3 the fluorescence intensity was markedly quenched by the addition of the polynucleotide. In contrast, no shifts of the emission maxima were observed.

By taking into account the very short singlet lifetime of both LOM (1 ns) and ENX (0.5 ns)[19] and the highest DNA concentration used (1.2×10^{-3} M), it appears evident that dynamic mechanisms involving the excited singlets of FQs cannot be the main quenching processes. Thus, it is more likely that static quenching mechanisms due to the interaction of ct-DNA with FQs in the ground state play a dominant role in the fluorescence quenching. In light of this, when the existence of different types of fluorophore-DNA complexes is considered, with association constants K_i and relative fluorescence quantum yields Φ_i, the ratio of fluorescence intensities in the absence (I_0) and in the presence (I) of ct-DNA is given by the following equation:[28]

$$I_0/I = (1 + \Sigma K_i[\text{ct-DNA}])/(1 + \Sigma \Phi_i K_i[\text{ct-DNA}]) \quad (2)$$

Since our results show that the fluorescence of the molecules bound to DNA is much lower than that of the free chromophores, we can assume that the FQs-ct-DNA complexes are basically nonfluorescent. As a consequence, eqn. (2) reduces to the familiar Stern–Volmer equation:

$$I_0/I = 1 + K_b[\text{ct-DNA}] \quad (3)$$

where $K_b = K_{SV} = \Sigma K_i$ is relative to the different types of complexes not distinguishable by fluorescence.[29] The values of K_b obtained from the linear plots in 10^{-3} M buffer were $1.3 \times 10^3 \pm 100$ and $3.2 \times 10^3 \pm 250$ M^{-1} for LOM and ENX, respectively (Fig. 4). In 10^{-2} M buffer, K_b of LOM and ENX decreased and was the same for both compounds, $4 \times 10^2 \pm 50$ M^{-1}. All these data are in very good agreement with those obtained by absorption titration and lend credibility to these measurements as well as to the model proposed.

Fluorescence sensitization by DNA bases as well as fluorescence polarization measurements represent other powerful tools to investigate the binding of chromophores to DNA. Since the excited singlet energies of all the four DNA bases are much higher[30] than those of FQs,[19] the selective excitation of DNA could sensitize the FQs fluorescence *via* potential singlet-singlet energy transfer processes. However, no sensitized fluorescence was observed in our case. Moreover, fluorescence polarization measurements in the presence of 10^{-3} M ct-DNA did not show any significant increase of the weakly polarized fluorescence ($p \approx 0.008$) found in aqueous medium for both FQs. These results are further in according to the proposal of non-fluorescent FQs-DNA complexes.

Laser flash photolysis experiments

The formation of FQ-ct-DNA complexes was also demonstrated by means of nanosecond laser flash photolysis experiments. We have recently demonstrated that in the case of ENX the lowest excited triplet state is produced very efficiently; it is characterized by an absorption with a maximum around 520 nm and the related lifetime is strongly and unusually dependent upon the concentration of the phosphate buffer.[15,19] As shown in Fig. 5, addition of increasing amounts of ct-DNA to

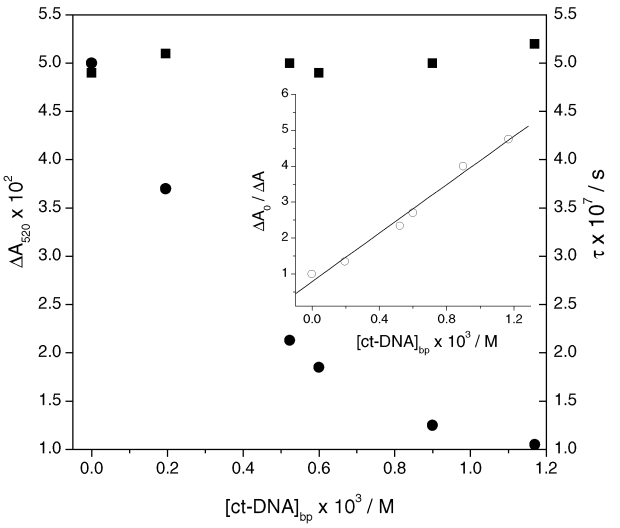


Fig. 5 Absorbance changes (●) (left axis) upon excitation with 20 ns, ~3 mJ laser pulse at 355 nm, taken 100 ns after pulse and lifetime (■) (right axis) related to the triplet of ENX observed in an argon-saturated ENX solution in 10^{-3} M phosphate buffer at pH 7.4 as a function of ct-DNA concentration. The inset shows the dependence of the ratio between the ENX triplet absorbance monitored at 520 nm (ΔA_0) in the absence and (ΔA) in the presence of ct-DNA as a function of the ct-DNA concentration.

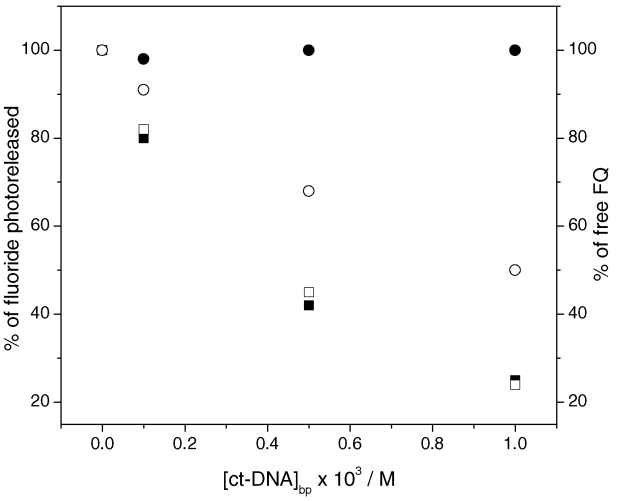


Fig. 6 Dependence of the % of fluoride photoreleased from nitrogen-saturated solutions of 10^{-4} M (●) LOM and (■) ENX in 10^{-3} M phosphate buffer at pH 7.4 on ct-DNA concentration (left axis). On the right axis the % of free (○) LOM and (□) ENX as a function of ct-DNA concentration is reported.

ENX solutions in 10^{-3} phosphate buffer led to significant reduction of the triplet-triplet transient absorption. In contrast, no changes in the lifetime (Fig. 5) or in the shape of the triplet-triplet absorption band were observed upon addition of ct-DNA. Interestingly, the plot of the ratio $\Delta A_0/\Delta A$ (where ΔA_0 and ΔA are the triplet absorption in the absence and in the presence of DNA, respectively) as a function of ct-DNA concentration (inset Fig. 5) gave a slope of $3.3 \times 10^3 \pm 300$ M^{-1}, in very good agreement with that obtained by fluorescence quenching experiments (see above).

The observed behavior reflects directly the formation of the ground state complex between ENX and ct-DNA. Indeed, the formation of such species precludes any significant population of the triplet, such that only unbound triplet is observed.

Increase of the buffer concentration to 10^{-2} M showed qualitatively similar results. On the other hand, from a quantitative point of view the quenching of the triplet absorbance was much less efficient and also in this case the value of the slope of the plot $\Delta A_0/\Delta A$ as a function of ct-DNA concentration (slope $= 5 \times 10^2 \pm 80$ M^{-1}) accorded with that obtained by fluorescence data in 10^{-2} M buffer.

Unfortunately, the very low intersystem crossing quantum yield of LOM as well as the optical transparency of other transient intermediates involved in its photodecomposition[19] precluded any type of laser flash photolysis measurements for this compound.

Photodefluorination in the presence of ct-DNA

The photodefluorination of LOM and ENX was investigated in the presence of different amounts of ct-DNA by following the release of fluoride ion through ^{19}F NMR spectroscopy. Fig. 6 shows the relative percentage of fluoride released as well as the percentage of the unbound FQ as a function of the polynucleotide concentration. It may be noted that the photodefluorination of LOM was basically unaffected by the presence of ct-DNA, indicating that the LOM-ct-DNA complex is characterized by a photoreactivity comparable to that of the free molecule. On the other hand, in the case of ENX, the photorelease of fluoride was strongly dependent on the formation of the ENX-ct-DNA complex. In particular, a very nice correspondence between the percentage of photodefluorination and the percentage of free drug was observed. This finding provides strong evidence that the photodefluorination of the ct-DNA-ENX adduct is much lower than that of the free FQ.

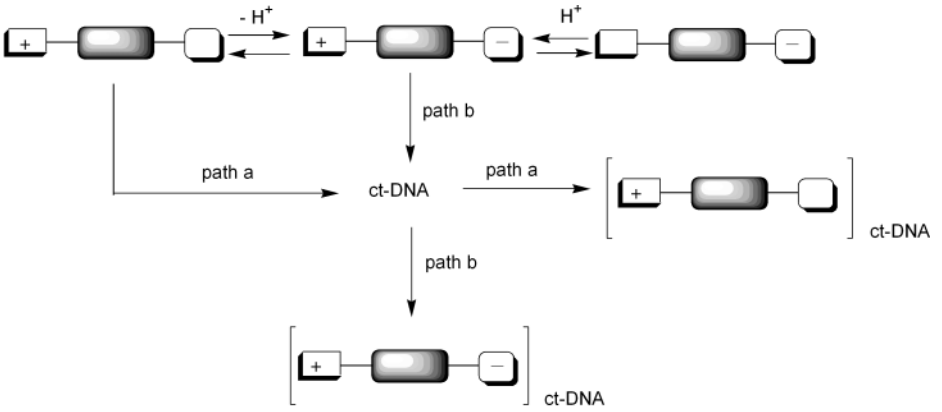
Discussion

Ground-state association of FQs to ct-DNA

The combination of several spectroscopic techniques shows that both LOM and ENX bind to ct-DNA. The binding efficiency is dependent on the buffer concentration as confirmed by the decrease of K_b upon going from 10^{-3} to 10^{-2} M phosphate. These results allow some insight to be gained on the binding mode of FQs to ct-DNA. According to the literature, decrease of the binding constant associated with an increase of the ionic strength is in general observed for typical cationic intercalator drugs.[31–34] This is due to the fact that the repulsive interactions between neighboring anionic phosphate groups are diminished at higher ionic strength. In these conditions intercalative insertion of molecules is expected to be less favored. However, the K_b values obtained for LOM and ENX in 10^{-3} M buffer are lower than those usually observed for classic intercalators. Therefore, a binding mode consisting of partial intercalation, though with significant FQs base-stacking interactions, could be conceivable. Spectroscopic and photochemical data are consistent with this hypothesis (see below).

At this point it is appropriate to recall briefly that due to the ionization of the piperazinyl ($pK_a \approx 8.3$) and carboxyl ($pK_a \approx 6$) groups both FQs investigated can be present in three different forms dependent on pH (see Scheme 1) and characterized by fairly different absorption spectra.[15,35] Such spectra are reported in Fig. 7 for the sake of clarity.

Given that for both LOM and ENX the dominant form at neutral pH is the zwitterionic one,[15,35] at first sight the model of a partial insertion would seem quite surprising. Indeed, by taking into account the presence of the negatively charged carboxyl group in these prototropic species, the insertion of the chromophoric skeleton of the molecules into the hydrophobic interior of DNA appears highly unlikely due to obvious coulombic reasons. However, further inspection of the absorption spectra reported in Fig. 1 reveals that the absorption of the



Scheme 1

bound forms of LOM and ENX are very similar to those of the cationic forms of FQs in which the carboxylic group is protonated[15,35] (see Fig. 7 for comparison). Such a behavior resembles that observed by Son *et al.* in the case of norfloxacin,[23] a FQ characterized by a molecular structure similar to that of LOM and ENX. In that case it has been suggested that protons promote FQ binding to DNA at neutral pH by neutralizing the negative charge on the carboxylate group in the bound drug.[23] However, we believe that in our case the reason for the behavior observed may lie in the almost exclusively selective interaction of the biopolymer with the cationic species of both FQs, making it the most abundant one at neutral pH as pictorially depicted in Scheme 1 (path a). This scenario presents close analogies to what we have recently found when LOM is in the presence of negatively charged sodium dodecylsulfate micelles at neutral pH.[36] Based on the molecular structure of the cationic forms of the FQs, a binding mode consisting of the positively charged piperazinyl group anchored to the negatively charged DNA surface and the neutral aromatic chromophore protruding towards the DNA interior can be reasonably proposed for these complexes.

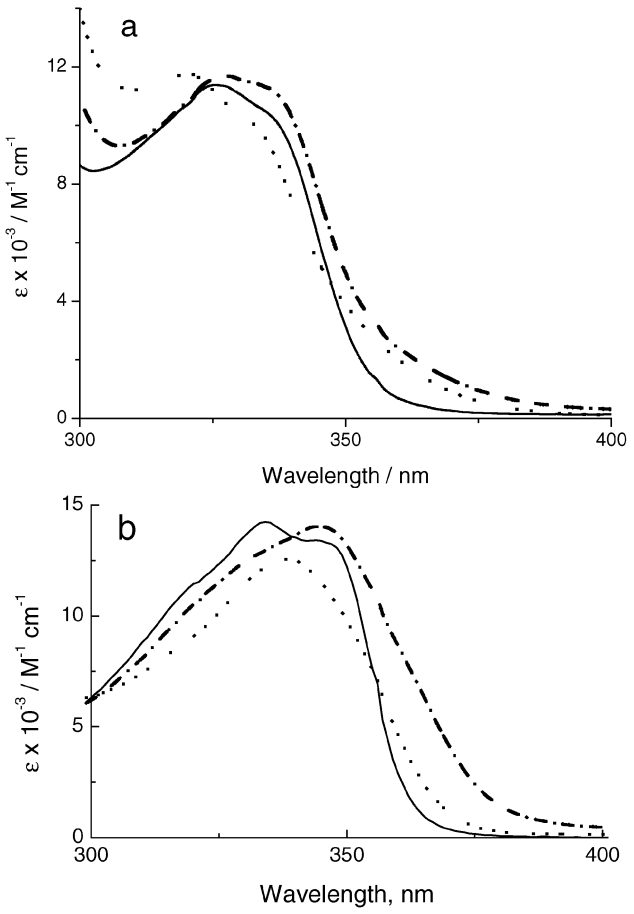


Fig. 7 Absorption spectra related to the (—) zwitterionic, (···) cationic and (·-·-·) anionic forms of (a) LOM and (b) ENX. Cell path: 1 cm.

This picture is corroborated by the significant hypochromic effect, broadening of the band and red shift of the bound species (Fig. 1, spectra 4) if compared to the cationic form of free LOM and ENX (Fig. 7). All of these effects are commonly accepted to provide confirmation of the proximity of small molecules to the DNA core.[27,37,38] In particular, hypocromism has been extensively suggested to be due to a strong interaction between the electronic states of the intercalated chromophore and those of the DNA bases.[37] The strength of this interaction is expected to decrease as the cube of the distance between the chromophore and the DNA bases. Thus, the effects observed in our case are consistent with a close proximity of the FQs to the DNA interior. The proposed model is in good agreement with the recent studies of Son *et al.*[23] on norfloxacin. It has been elegantly demonstrated that the molecular plane of norfloxacin is near perpendicular relative to the DNA helix axis and therefore potentially suitable to allow insertion of the molecule into the DNA interior.[23] The low value of K_b obtained for LOM compared to ENX is in good agreement with the molecular structure of the two compounds. Indeed, due to the presence of the additional fluorine atom in the 8 position, LOM is expected to be less favored than ENX for a deep insertion into the hydrophobic region of the DNA.

Further evidence for proximity of the FQs to the DNA core in 10^{-3} M buffer was provided by the ICD spectra. As shown in Fig. 2, negative ICD bands with absorption peaks corresponding fairly well to those of the absorption bands were observed for both LOM and ENX. In general, ICD signals are also dependent on the cube of the distance of the two interacting partners and are commonly observed for molecules closely bound to the helix due to their asymmetric environment.[39]

Increase of the buffer concentration by one order of magnitude leads to a significant decrease of the association constants, which reach basically the same value for both FQs. This result, along with the slight absorbance changes observed even at high ct-DNA concentrations, suggested that a different mode of binding probably occurs under these experimental conditions. As specified above, increase of the ionic strength diminishes the repulsive interactions between neighboring anionic phosphate groups of the DNA backbone, making the insertion of the chromophores into deep regions of the helix

highly disfavored. We believe that because of this effect, the accessibility of the cationic form of FQs to the DNA interior is not feasible. The dominant binding mode occurring in 10^{-2} M phosphate buffer appears most likely to be an external complex in which the positively charged piperazinyl group is anchored to the negatively charged DNA helix and the aromatic chromophore is protruding towards the aqueous bulk solution. In particular, by considering that the absorption spectra of the FQs observed in the presence of DNA (spectra not shown) are very similar to those of the zwitterionic forms, an interaction involving such a species is believed to be the dominant one (path b in Scheme 1). The hypothesis of external complexes characterized by the structure proposed above is corroborated by the similarity of the K_b values obtained for LOM and ENX in 10^{-2} M phosphate buffer. Such a result indicates that the key interaction could mainly involve the piperazinyl group, whose structure is quite similar for both FQs, and only to a minor extent the chromophoric part of the molecules. It is worth noting that a similar interaction of the positively charged $>N(4')H_2^+$ moiety of ENX and LOM with phosphate ions of the buffer was pointed out in our recent study.[19] The association constants for these complexes with the phosphate buffer were more than one order of magnitude lower than those obtained here for ct-DNA. These differences might be reasonably attributed to the higher electrostatic charge density distributed around the DNA helix.[38]

The formation of the proposed external complex finds further support in the absence of ICD signals when 10^{-2} M phosphate buffer is used, even at DNA concentrations higher than 10^{-3} M, in which a remarkable amount of complex is present. This observation is in agreement with a large separation between the chromophoric portion of the FQs and the asymmetric environment of the DNA structure, ruling out significant localization of the chromophoric skeleton either in the major or minor grooves of ct-DNA. Indeed, as is well-known the magnitude of the ICD signal is expected to decrease as the cube of the distance between the chromophore and the DNA bases.[39]

Fluorescence quenching

ct-DNA quenched the fluorescence of both FQs but no shift in the emission maxima was observed (Fig. 4). By taking into account that the singlet lifetimes of both FQs are very short ($\leqslant$1 ns)[19] and that the highest concentration of ct-DNA concentrations used in the fluorescence experiments was 1.2×10^{-3} M, it is possible to exclude any involvement of a diffusion-controlled mechanism in the fluorescence quenching since $k_{diff}[\text{ct-DNA}] \ll 1/\tau_{S1}$. Static quenching due to the interaction of the DNA with FQs in the ground state is therefore the most probable process. The shapes of the excitation spectra for LOM and ENX in the presence of DNA were the same as those of the free chromophore, confirming that the FQs-DNA complexes are basically nonfluorescent. This fact accounts well for the apparent incongruity emerging from the fluorescence polarization measurements. Indeed, an increase in the degree of polarization is commonly expected for molecules embedded into the DNA structure.[37] In our case, the lack of any increase in the degree of polarization can be due to the fact that the polarization observed in the presence of the biopolymer is always related to the fraction of unbound FQs. Finally, the formation of nonfluorescent FQs-ct-DNA complexes also explains the lack of sensitized fluorescence observed upon selective excitation of the DNA bases at 260 nm.

We believe that the fluorescence quenching within the FQs-ct-DNA complexes in the case of low buffer concentration could more probably occur through a very fast electron transfer involving the DNA bases, given their close proximity to the excited chromophores. This proposal is supported by thermodynamic considerations. Indeed, by taking into account that the reduction potential for FQs (the electron acceptors, A) is *ca.* −1.1 V *vs.* NHE,[40] for the DNA bases (the electron donors, D) is $\leqslant$1.4 V *vs.* NHE[41] and that the singlet energy for FQs ($\Delta E_{0,0}$) is close to 3.5 eV,[19] it can be noticed that the thermodynamic balance, based on the Rehm–Weller equation,[42]

$$\Delta G^0 = E^0(D^+/D) - E^0(A^-/A) - \Delta E_{0,0} \quad (4)$$

is negative, making the photoinduced electron transfer highly feasible.

As proposed in the case of 10^{-2} M buffer (see above), absorption spectral changes and ICD measurements are consistent with the formation of FQs-ct-DNA external complexes in which the chromophoric unit of the FQs is quite distant from the DNA bases and mainly exposed to an aqueous environment. Such a separation of the FQs from the DNA interior reduces, of course, the possibility for fluorescence quenching triggered by a photoinduced electron transfer between the excited FQs and the DNA bases. Thus, a different fluorescence quenching mechanism within the FQs-ct-DNA complexes is thought to take place. We believe that the ground state association with DNA might preclude fluorescence, either by increasing the internal charge transfer interaction in the singlet excited state and channeling the energy into a radiationless path or by promoting the deprotonation of the nitrogen atom in the excited state through the action of the closely located phosphate stem, which acts as a proton acceptor. A lower basicity of the piperazynyl N(4′) in the excited state is indeed expected on the basis of the pH dependence of the FQs absorption spectra.[15,36] Such a fluorescence quenching mechanism is not unprecedented. In fact, it presents close analogies to what was recently found in the case of both FQs-phosphate buffer complexes[19] and other supramolecular structures in which phosphate ions and positively charged amino groups interact with each other in the ground state.[43]

Photoreactivity of the FQ-ct-DNA complexes

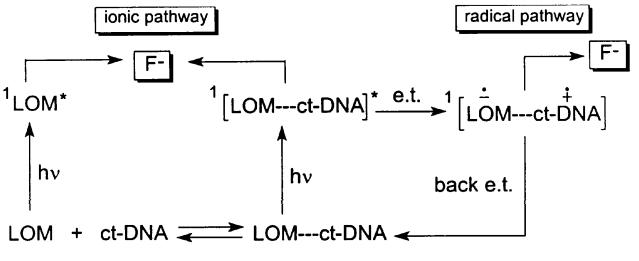
As shown in Fig. 6, the photodefluorination of the two FQs shows a differentiated behavior upon addition of ct-DNA. In fact, the photorelease of fluoride in the case of LOM was basically unaffected by the presence of increasing amounts of biopolymer. On the contrary, the relative efficiency of fluoride photorelease from ENX strongly decreased with ct-DNA addition.

In order to understand such different photobehavior for the two complexes we consider it useful, for the sake of clarity, to recall again briefly the main features regulating the photodefluorination processes of the two FQs in aqueous solution. In this medium LOM undergoes efficient loss of fluoride mediated mainly by its lowest singlet excited state. This singlet photoreactivity is necessarily related to the intramolecular charge transfer character of this state.[12–14,19] The presence of the two electron-withdrawing fluorine atoms plays a key role in the photoreactivity of LOM, making the heterolytic cleavage of the C–F bond fast enough to compete with intersystem crossing. On the other hand, in the case of ENX the fluoride photorelease is triggered almost exclusively by the lowest excited triplet state.[15,19] Interestingly, both the efficiency and nature of photostimulated ENX defluorination are strongly dependent on the presence of phosphate buffer, which drives the photodegradation from an ionic to a radical pathway.[11,19] It has been demonstrated that this uncommon effect is due to an unexpected and surprising bimolecular electron transfer involving the lowest triplet state of ENX as electron acceptor and the phosphate ions of the buffer as electron donor.[19] The results displayed in Fig. 6 accord well with the picture proposed about partial intercalation of the FQs into the DNA

interior as well as with the intrinsic photochemical properties of the free molecules (see below).

In the case of LOM, despite fluorescence being efficiently quenched by DNA the photodefluorination was not influenced, suggesting that the photoreactivity of the complex is comparable to that of the free FQ. Two possible mechanisms can be conceived to account for this behavior (Scheme 2). (*i*) The photodefluorination process in the complex occurs as fast as in the unbound molecule so that it is highly competitive with the photoinduced electron transfer between the excited LOM and the DNA bases. Therefore, such a static mechanism responsible for the fluorescence quenching would play only a minor role in the deactivation of the singlet state. In this case the photodefluorination will follow again an ionic mechanistic pathway as observed in the absence of DNA. (*ii*) On the contrary, if the photoinduced electron transfer between excited LOM and DNA bases responsible for the fluorescence quenching plays a major role, the insensitivity of the fluoride photorelease to DNA complexation would suggest that photodefluorination has to be highly competitive with the back electron transfer process. This hypothesis is well-supported by the fact that release of the fluoride anion from an environment characterized by a strong negatively charged surface due to the phosphate moieties is expected to be highly favored due to coulombic effects.[44] In this case, of course, the photodefluorination mechanisms should follow a radical pathway given that it would be mediated by a radical anion intermediate. This view is corroborated by the recent results obtained by Spratt *et al.*[45] It has been indeed pointed out that upon UVA irradiation LOM leads to the formation of DNA base oxidation photoproducts originating from radical pathways.[45] In light of these data and by taking also into account the favorable thermodynamic balance reported above, the participation of a photodefluorination pathway according to mechanism (*ii*) would seem to be the most plausible.

As far as the ENX-DNA complex is concerned, it can be noted that the photodefluorination efficiency is strictly dependent on the amount of complex formed for a given ct-DNA concentration. In particular, the good correlation between the percent of photodefluorination and the percent of free FQ shown in Fig. 6 is consistent with the fact that the observed photodefluorination is basically due only to the fraction of unbound ENX. Thus, the photoreactivity of the complex can be considered negligible. This trend accords well with the triplet photoreactivity of the free ENX and finds a logical explanation in the laser flash photolysis experiments performed in the presence of ct-DNA. Indeed, as displayed in Fig. 5 ground state complexation greatly reduces the yield of the ENX triplet. This result is attributed to static quenching of the excited singlet state of the bound ENX, which precludes significant population of the photoreactive triplet state. In this case, the negligible photoreactivity of the ENX-ct-DNA adduct suggest that if static quenching of the ENX singlet state is due to a photoinduced electron transfer with the DNA bases as proposed above, the back electron transfer route strongly dominates over the defluorination pathway. This picture is in accordance with the negligible singlet photoreactivity shown by ENX in the absence of DNA.[14,15,19] However, by taking into account that our previous study has demonstrated that the cationic form of ENX is characterized by a much lower triplet yield than the zwitterionic one,[15] it should be considered that the triplet yield reduction observed in the case of the complex could be also due to the preferential binding of the cationic ENX to DNA as demonstrated by the absorption studies (see above).



Scheme 2

As shown in Fig. 5, addition of ct-DNA did not lead to any significant changes in the triplet lifetime of ENX. Since the signals observed are due only to the unbound chromophore, we can exclude the involvement of dynamic quenching processes involving the DNA phosphate backbone and the ENX triplet. This result is quite surprising. Actually, in light of the active role played by the phosphate ions of the buffer in quenching the triplet state of ENX *via* electron transfer,[19] a direct involvement of the phosphate ions of the DNA backbone as potential quenchers of the ENX triplet would have been expected. A possible explanation for the ineffectiveness of such a process might be due to the weaker reductive power of the DNA phosphate monoanions with respect to the HPO_4^{2-} dianion of the buffer.[19,46]

Conclusions

The present work has demonstrated that both the mode and efficiency of binding of the photocarginogenic and phototoxic FQs to calf thymus DNA depends on the buffer concentration. In 10^{-3} M phosphate buffer, ct-DNA interacts preferentially with the cationic form of LOM and ENX. Both compounds seem to be located in close proximity to the DNA bases and a partial intercalation into the DNA interior appears highly probable. A one order of magnitude increase of the buffer concentration leads to a displacement of the molecules from the DNA interior and pushes their chromophoric part out towards a mainly aqueous environment. External complexes, characterized by lower association constants than those observed in the more dilute buffer and involving mainly the zwitterionic form of the FQs, are formed under these conditions. The positively charged piperazinyl ring of LOM and ENX is thought to behave as a pivot, going from one binding mode to another. In fact, in both cases it can interact with the negatively charged phosphates of the DNA stem *via* a highly favored electrostatic interaction.

The FQs-ct-DNA complexes are characterized by fairly different photoreactivities. While the LOM-ct-DNA complex reacts with an efficiency similar to that of the unbound LOM, the complexation of ENX with the biopolymer leads to a dramatic decrease of the photodefluorination yield.

Beyond contributing to the general picture of drug photoprocesses in organized assemblies, we believe that the results presented herein may contribute to an understanding of the molecular basis of the still complex issue concerning the photocarcinogenic and phototoxic effects induced by these drugs. It may be anticipated that the higher capability of LOM in inducing DNA breakage upon UVA irradiation with respect to ENX [3,4] is related to the high photodefluorination efficiency of LOM when compared to ENX in aqueous medium.[11] However, the present investigation suggests that both binding and photoreactivity of the FQs-DNA complexes are key parameters that should be taken into account in order to gain a more adequate picture concerning the photosensitizing effects induced by these molecules. In this connection, it should to be stressed that: (1) the binding of these FQs to ct-DNA occurs without requiring any mediation by Mg^{2+} ions as a bridge between the phosphate groups of the biopolymer and the carboxyl moieties of FQs;[47] and (2) the highly efficient DNA cleavage photoinduced by LOM can be related to the fact that the drug molecules bound to the bio-target are as photoreactive as the free ones.

As a consequence, in the case of LOM the main transient species responsible for DNA damage are photogenerated in the vicinity of the helix and thus attack at the target can be highly competitive with other deactivation pathways of these primary photolysis products. In contrast, in the case of ENX only the unbound molecules are photoreactive and thus, the transient intermediates photogenerated after photodefluorination need to diffuse towards DNA prior to reaction with this target. Under these conditions, the fraction of active species responsible for DNA breakage can be significantly lower than that produced immediately after photodefluorination since deactivation channels competitive with the DNA reaction may play a non-negligible role. This view is consistent with the large variety of photobiological data available on the topic.

Experimental

Materials

Lomefloxacin hydrochloride and enoxacin were purchased from Sigma Chemicals Co. (Milan). Sonicated calf thymus DNA (phenol extracted, lyophilized, average size 2000 bases, range 200–6000 bases) was obtained from Pharmacia (Milan). The concentration of DNA in base pairs ($[ct\text{-}DNA]_{bp}$) was determined spectrophotometrically. Water was purified through a Millipore Milli-Q system. All the experiments were performed in either 10^{-3} or 10^{-2} M phosphate buffer at pH 7.4. The pH of the solutions was measured with a glass electrode.

Spectroscopic measurements

Absorption and induced circular dichroism spectra were recorded with a Beckman 650 DU spectrophotometer and a Jasco J-615 dichrograph, respectively. Fluorescence emission spectra and fluorescence polarization measurements were recorded with a Spex Fluorolog-2 (model F-111) spectrofluorimeter. The ^{19}F NMR spectra were recorded on a Varian Unity Inova spectrometer at 470.30 MHz, operating at a temperature of 27 °C with a spectral width of 20 KHz (42.5 ppm), an acquisition time of 2 s, a flip angle of *ca.* 40° and an interpulse delay of 2 s; exponential line broadening of 1 Hz and baseline correction were used in free induction decay (FID) processing. KF (0.01 M in buffer solution at pH 7.4 with 20% D_2O) was used as external reference, setting its resonance at 0.00 ppm.

Irradiation conditions

Irradiation of FQs in the presence of DNA was performed using monochromatic light obtained from a Series 200 He-Cd 325 nm laser (Liconix, Santa Clara CA, USA). All the samples were degassed for 30 min with nitrogen before irradiation. The incident photon flux on 3 mL quartz cuvettes was *ca.* 5×10^{15} quanta s^{-1}. The experimental procedures of irradiation and the light intensity measurements have been described previously.[48,49] The amount of fluoride photoreleased was in all cases determined for up to 12% transformation of the starting compounds.

Nanosecond laser flash photolysis

The set-up for the nanosecond absorption measurements has been described previously.[50] The laser beam (Nd-YAG, JK Lasers, pulse 20 ns FWHM, 355 nm) was focused on a 3 mm high and 10 mm wide rectangular area of the cell and the first 2 mm were analyzed in a right angle geometry. The energy used was $\leqslant$4 mJ $pulse^{-1}$. Spectral resolution was 2 nm. The sample absorbance was *ca.* 0.3 at 355 nm for a 1 cm pathlength. Oxygen was removed by vigorously bubbling the solutions with a constant flux of argon, previously passed through a water trap to prevent evaporation of the sample. Care was taken to renew the solution at each laser shot.

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