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Original Contribution

Seeking the mechanism responsible for fluoroquinolone photomutagenicity: a pulse radiolysis, steady-state, and laser flash photolysis study

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ABSTRACT

The mechanism responsible for the remarkable photomutagenicity of fluoroquinolone (FQ) antibiotics remains unknown. For this reason, it was considered worthwhile to study in detail the interactions between DNA and a dihalogenated FQ such as lomefloxacin (LFX; one of the most photomutagenic FQs) and its *N*-acetyl derivative ALFX. Studies of photosensitized DNA damage by (A)LFX, such as formation of DNA single-strand breaks (SSBs), together with pulse radiolysis, laser flash photolysis, and absorption and fluorescence measurements, have shown the important effects of the cationic character of the piperazinyl ring on the affinity of this type of drug for DNA. Hence, the formation of SSBs was detected for LFX, whereas ALFX and ciprofloxacin (a monofluorated FQ) needed a considerably larger dose of light to produce some damage. In this context, it was determined that the association constant (K_a) for the binding of LFX to DNA is ca. $2 \times 10^3\ M^{-1}$, whereas in the case of ALFX it is only ca. $0.5 \times 10^3\ M^{-1}$. This important difference is attributed to an association between the cationic peripheral ring of LFX and the phosphate moieties of DNA and justifies the DNA SSB results. The analysis of the transient species detected and the photomixtures has allowed us to establish the intermolecular processes involved in the photolysis of FQ in the presence of DNA and 2′-deoxyguanosine (dGuo). Interestingly, although a covalent binding of the dihalogenated FQ to dGuo occurs, the photodegradation of FQ…DNA complexes did not reveal any significant covalent attachment. Another remarkable outcome of this study was that (A)LFX radical anions, intermediates required for the onset of DNA damage, were detected by pulse radiolysis but not by laser flash photolysis.

Fluoroquinolones (FQs)[1] are molecules formed by a quinolinic main ring and an aminoalkyl substituent. They are widely used as antibacterial agents that develop their pharmacological activity through the inhibition of a bacterial gyrase enzyme (topoisomerase II) involved in the replication and repair of bacterial DNA [1]. During the past years, FQs have received much attention owing to their antitumoral activity [2–6]. In vitro and in vivo studies have confirmed their anti-cancer effects, supported by the reduction in all-cause mortality among cancer patients [7]. The direct FQ antitumor effect has been associated with the inhibition of mammalian deoxyribonucleic acid topoisomerase I, topoisomerase II, and DNA polymerase. Moreover, the genotoxic effects exhibited by FQs in eukaryotic systems are enhanced by UV irradiation [8], which confers on them a potential property as photochemotherapeutic agents. This photoinduced genotoxicity has remarkably been detected in 6,8-dihalogenated FQs such as fleroxacin, BAY y3118, and lomefloxacin (LFX; compound proposed in the literature as a photomutagenic standard, see Chart 1) [9–16]. In this context, a large number of studies concerning the photophysical and photochemical properties of a 6,8-dihalogenated FQ have been carried out during the past few years [12,17–21]. Most of them have shown an unusual photodehalogenation by heterolysis of the strong C_8–halogen bond from their triplet excited states (^{3}FQ) [17–21]. This process leads to the generation of an aryl cation with alkylating properties [17–21]. Therefore, the photoinduced DNA damage has been associated with the reactivity of this intermediate [9,19,21]. This is based on the observation of the quenching of an aryl cation arising from ^{3}LFX photodehalogenation by guanosine monophosphate (dGMP) and the detection of a covalent

Abbreviations: ALFX, *N*-acetyllomefloxacin; CFX, ciprofloxacin; dGMP, 2′-deoxyguanosine 5′-monophosphate; dGuo, 2′-deoxyguanosine; FM, flumequine; FQ, fluoroquinolone; LFX, lomefloxacin; PB, phosphate buffer; SSB, DNA single-strand break.

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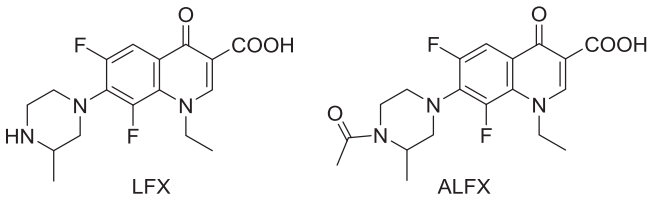

Chart 1. Structure of LFX and its *N*-acetyl derivative ALFX.

binding between LFX and this nucleotide (LFX–dGMP) [21]. However, studies concerning the association of LFX with DNA have revealed that an electron transfer reaction between the singlet excited state of complexed LFX (^{1}LFX…DNA) and DNA must also be involved in the photodehalogenation because, despite the important decrease in LFX emission, the efficiency of this process does not change in the presence of increasing amounts of DNA [22]. Thereby, the literature findings suggest that ^{1}LFX…DNA and ^{3}LFX might be candidates for covalent binding of LFX to DNA.

With this background, the *main processes* involving *LFX photodegradation in the presence of* 2′-deoxyguanosine (*dGuo) or DNA were evaluated by performing* emission studies, laser flash photolysis, pulse radiolysis, and product analysis using ultraperformance liquid chromatography with high-resolution mass spectrometry detection (UPLC–HRMS). In this context, DNA photodamage was assessed through the detection of single-strand breaks in plasmid pBR322 and by examination of the UV–Vis absorption and fluorescence changes in DNA after its photosensitization with FQ and subsequent separation by gel-filtration chromatography to investigate photobinding of LFX to DNA.

Moreover, as it has been established that acetylation of the piperazinyl ring of FQs produces changes in their photophysical and/or photochemical behavior [17,23–25], some key experiments were also performed using the lomefloxacin acetylated derivative 7-(4-acetyl-3-methyl-1-piperazinyl)-1-ethyl-6,8-difluoro-1,4-dihydro-4-oxoquinoline-3-carboxylic acid (ALFX).

Materials and methods

General materials

Calf thymus DNA, ciprofloxacin (CFX), dGuo, flumequine (FM), and LFX were commercial products obtained from Sigma–Aldrich, whereas plasmid pBR322 was supplied by Roche and Sephadex G-25 columns by GE Healthcare. Sodium phosphate buffer (PB) and sodium bicarbonate buffer were prepared from reagent-grade products using Milli-Q water; the pH of the solutions was measured through a glass electrode and adjusted with NaOH to pH 7.4. Other chemicals were of reagent grade and used as received.

The samples of FQs were prepared with various PB concentrations starting from a stock solution of 300 mM PB adjusted to pH 7.4. ALFX was prepared as previously described from a solution of LFX (300 mg, 0.9 mmol) in Ac_2O (50 ml) that was refluxed for 7 h [17]. The solution was cooled to room temperature and concentrated. Afterward, the residue was dissolved in water, neutralized to pH ~7.4, extracted with CH_2Cl_2, and concentrated to dryness.

Absorption and emission measurements

Ultraviolet spectra were recorded on a UV–Vis scanning spectrophotometer (Cary 50). Fluorescence emission spectra were recorded on a Photon Technology International (PTI) LPS-220B fluorimeter. Lifetimes were measured with a time-resolved spectrometer (TimeMaster fluorescence lifetime spectrometer TM-2/2003) from PTI by means of the stroboscopic technique, which is a variation of the boxcar technique. A hydrogen/nitrogen flash lamp (1.8-ns pulse width) was used as excitation source. The kinetic traces were fitted with monoexponential decay functions. Measurements were done under aerated conditions at room temperature (25 °C) in cuvettes of 1-cm path length. The excitation wavelength used to register the fluorescence lifetime was 320 nm. The fluorescence quantum yield of quinine bisulfate in 1N H_2SO_4 (ϕ_F=0.546) was used as standard.

Fluoroquinolone fluorescence quenching by DNA after excitation at 355, 348, and 330 nm was performed using 10^{-4} M FQ buffered aqueous solutions (10^{-3} M PB, pH ~7.4). The DNA concentrations were determined spectrophotometrically taking into account a molar extinction coefficient $\varepsilon_{258\ nm}$=6700 $cm^{-1}\,M^{-1}$ [22,26]. *Eq.* (1) was selected to determine the drug–DNA interactions from fluorescence quenching data [27–31]:

$$F_0/F = 1 + K_{sv}[Q], \quad (1)$$

where F_0 and F are the fluorescence intensities in the absence and presence of the quencher, respectively; [Q] is the quencher concentration (DNA from 10^{-5} to 1.5×10^{-3} M in nucleotides); and K_{sv} is the Stern–Volmer quenching constant.

Laser flash photolysis experiments

A pulsed Nd:YAG laser was used for the excitation at 355 nm. The single pulses were ~10 ns duration and the energy was from 10 to 1 mJ/pulse. A pulsed xenon lamp was employed as detecting light source. The laser flash photolysis apparatus consisted of the pulsed laser, the Xe lamp, a monochromator, and a photomultiplier made up of a tube, housing, and power supply. The output signal from the oscilloscope was transferred to a personal computer.

Aqueous solutions of 10^{-4} M (A)LFX were prepared in 10^{-3} M $NaHCO_3$ and the experiments registered under anaerobic conditions bubbling N_2O. Transient absorption spectra at different times after the laser pulse were obtained for each sample in the presence and the absence of DNA, paying special attention to intersystem crossing quantum yield changes and to the generation of new intermediates. The DNA concentrations ranged between 10^{-4} and 10^{-2} M in nucleotides.

The quenching experiments were carried out keeping the pH constant at 7.4 throughout the experiment.

Rate constants of aryl cation quenching by biomolecules were determined using the Stern–Volmer Eq. (2):

$$1/\tau = 1/\tau_0 + k[Q]. \quad (2)$$

Pulse radiolysis

The pulse radiolysis experiments were carried out with a 12-MeV Radiation Dynamics Ltd. (UK) 3-GHz electron linear accelerator. We used a single-pulse mode with a pulse duration from 0.22 to 2 μs and with a peak current of about 30 mA. The accelerator is normally operated at 10 pulses per second but the single-pulse mode is achieved by modifying the pulses to the gun [32]. The detection system consisted of a Xe arc lamp and a pulsing unit, high-radiance Kratos monochromator, and quartz optics. Optical transmissions at various wavelengths selected with the monochromator, bandwidths 10 nm, were observed as a function of time before and after the radiation pulse using photoelectric detection. The output of the photomultiplier (EMI 9558Q) was displayed on a Tektronix TDS 380 digitizing oscilloscope. Data processing was performed on a Dan PC using software developed in-house. The sample cell, constructed from Spectrosil quartz, had an optical path length of 25 mm [32].