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Structure–activity relationship and role of oxygen in the potential antitumour activity of fluoroquinolones in human epithelial cancer cells

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ABSTRACT

The photobehavior of ciprofloxacin, lomefloxacin and ofloxacin fluoroquinolones was investigated using several *in vitro* methods to assess their cytotoxic, antiproliferative, and genotoxic potential against two human cancer cell lines. We focused our attention on the possible relationship between their chemical structure, O_2 partial pressure and photobiological activity on cancer cells. The three molecules share the main features of most fluoroquinolones, a fluorine in 6 and a piperazino group in 7, but differ at the key position 8, unsubstituted in ciprofloxacin, a fluorine in lomefloxacin and an alkoxy group in ofloxacin. Studies in solution show that ofloxacin has a low photoreactivity; lomefloxacin reacts via aryl cation, ciprofloxacin reacts but not via the cation. In our experiments, ciprofloxacin and lomefloxacin showed a high and comparable potential for photodamaging cells and DNA. Lomefloxacin appeared the most efficient molecule in hypoxia, acting mainly against tumour cell proliferation and generating DNA plasmid photocleavage. Although our results do not directly provide evidence that a carbocation is involved in photodamage induced by lomefloxacin, our data strongly support this hypothesis. This may lead to new and more efficient anti-tumour drugs involving a cation in their mechanism of action. This latter acting independently of oxygen, can target hypoxic tumour tissue.

1. Introduction

Fluoroquinolones (FQs) are widely used broad-spectrum antibiotics that play an important role in the treatment of human and animal infections [1,2]. They are strong inhibitors of bacterial DNA gyrase and type II DNA topoisomerase in case of mammalian cells. These enzymes are responsible for DNA supercoiling and are involved in chromosome condensation, DNA replication, transcription and recombination [3,4], therefore, agents targeting them determine a strong inhibition of cell growth. FQs used clinically against bacterial infections can be administered *per os* at high doses, thanks to their relatively limited adverse effects [1]. However, some of them possess toxic or genotoxic potential when exposed to UVA irradiation [5–7]. A number of photochemical and photophysical studies, dealing with the mechanisms of photosensitization induced by these drugs, have shown that FQs photoreactivity may be modulated by the nature and position of the substituents attached to the quinolone ring [8–10]. The large majority of FQs contain a fluorine atom in position 6 of the condensed ring; in some cases, further fluorine atoms are present at 5 or 8 positions. It is generally assumed that adverse photoreactions *in vivo* caused by exogenous molecules are due to activation of oxygen to produce reactive oxygen species [11,12]. However, this is not an absolute rule and it has been suggested that in some cases, notably with some FQs, a reaction by aggressive intermediates formed in the photodegradation of the drug is involved [2,13,14]. FQs in fact are poor oxygen sensitizers, while exhibiting a varied photochemistry. As an example C_8 fluorinated FQs, such as Lomefloxacin (LFLX), undergo S_N1 heterolysis of the C_8—F bond to give the corresponding triplet aryl cation. This has been shown to react with DNA nucleotides, in particular with purine bases [15,16]. This reaction is oxygen-independent due to the short lifetime of the cation involved [13] and has been proposed as the main process operating in the genotoxic effects of LFLX, Fleroxacin, Sparfloxacin [6,17,18]. LFLX was also able to induce the development of malignant skin tumours in photo-treated mice [19,20].

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Studies in solution have clarified the chemistry of such drugs, but the link to the phototoxic and photogenotoxic activity of FQs remains to be investigated. Indeed, there is no evidence whether mechanisms, oxygen activation and photofragmentation might contribute to the overall toxic effect in the cell and to up which degree these mechanisms are involved. This would be important for devising drugs useful for an oxygen-independent phototherapy against cancer cells. In fact, fast growing tumours are often hypoxic due to insufficient vascularisation. Under such conditions, and in the presence of localized UVA radiation, a molecular reaction, e.g. heterolysis of the C_8—F bond, resulting in attack to DNA would represent a targeted therapy against cancer cells.

Accordingly, the present study aimed at investigating the chemical structure/O_2 partial pressure effect on the photobiological activity of Ciprofloxacin (CFLX), LFLX and Ofloxacin (OFLX) against two cancer epithelial cell lines. These three FQs share the main features of most FQs (a fluorine in 6 and a piperazino group in 7), but differ at the key position 8, non substituted in CFLX and bearing a fluorine in LFLX and an alkoxy group in OFLX (Fig. 1A). Studies in solution show that the latter compound has a low photoreactivity, CFLX reacts but not via the cation, LFLX reacts via the cation. In particular, the efficiency of the three FQs as potential anti-cancer agents was explored by assessing their toxic, anti-proliferative and pro-apoptotic effects on HeLa (cervical squamous carcinoma) and A431 (epidermoid carcinoma) cells, after UVA exposure. For this purpose, cell viability by tetrazolium derivative (MTS assay), cytofluorimetric analysis of the cell cycle by propidium iodide (PI) and apoptosis by Annexin test were performed. In addition, the photogenotoxic activity was assessed on both supercoiled DNA *in vitro* assay or by Comet test after phototreatment of the cultured cancer cell lines. Finally, the DNA-damage response of cells was evaluated by H_2AX activation. All these experiments have been performed at both normoxic and hypoxic environmental conditions in an attempt to correlate photobiological activity/chemical structure of each compound with the oxygen-independent or dependent mechanism of action.

2. Materials and methods

2.1. Chemicals

Lomefloxacin hydrochloride and Ofloxacin were obtained by Fluka-Sigma Aldrich, (Milan, Italy), while Ciprofloxacin hydrochloride was obtained from Santa Cruz biotechnology, (California, USA). All the chemicals used were dissolved in water.

2.2. Cell culture and treatments

HeLa S3 (HeLa) and A431 (kindly provided from L. Lanfrancone, IFOM-IEO, Milan) cell lines were grown in high glucose Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 10% foetal bovine serum (FBS, Gibco BRL), 2 mM L-glutamine (Gibco BRL), 100 U/ml penicillin, 100 μg/ml streptomycin in a 5% CO_2 atmosphere.

Cell treatments with CFLX, LFLX or OFLX were performed by incubating cells plus drug for 24 h. Negative controls consisted of non-irradiated cells and irradiated cells without drug. In all the figures, irradiated cells without drug are showed as control. For irradiation, cells were put in PBS. Cells were treated at physiological oxygen partial pressure and under hypoxic conditions. These latter were achieved as follows: Petri dishes with cells in PBS were put in an air tight UVA-transparent PET enclosure, saturated with N_2 for 30 min. PBS oxygen content, as measured with a WTW (Weilheim, Germany) OXI 340i oxymeter, was 10.7%, whereas the normal oxygen content of PBS kept in air was 20%. Cells were irradiated with a 16 W CAMAG (Muttenz, CH) UV-lamp emitting at 366 nm. Irradiation time was 26 min at 10 cm distance from the lamp, in order to achieve a 3.5 W/cm^2 UVA dose. UV-radiation intensity was measured at the cell surface level with an Ultraviolet Products (Upland Ca) UV-meter.

2.3. MTS cytotoxicity assay

HeLa or A431 cells were plated in 96-well tissue culture plates at the concentration of 1×10^4 cells/well, treated as described above at the drug concentrations of 25, 50 and 100 μg/ml. Cell viability was determined by the colorimetric assay (Promega) composed of the novel tetrazolium compound 3-[4,5-dimethylthiazol-2-yl]-5-(3-carboxymethoxyphenil)-2-(4-sulphonyl)-2H tetrazolium (MTS) inner salt, and an electron coupling reagent, phenazine ethosulphate (PES). The maximum non-toxic concentration of 50 μg/ml was used for all fluoroquinolones and for the most subsequent experiments.

2.4. Intracellular localization of FQs

In order to verify the cellular uptake of the FQs, cells were seeded on coverslips at the density of 5×10^4. After 24 h of treatment with each substance, cells were fixed in 2% formaldehyde for 5 min at room temperature (r.t.), and then post-fixed in 70% ethanol. After re-hydration, samples were washed in PBS and stained in PBS containing 0.5 μg/ml PI and 1 mg/ml RNase A.

Alternatively, treated-cells were incubated with Cell Light Lysosomes-GFP BacMam 2.0 (Invitrogen) or MitoTracker Red CM-H_2 XRos (Invitrogen) dye, staining lysosomes and mitochondria, respectively. Fluorescence signals were acquired with a TCS SP5 II Leica confocal microscope, at 0.3 μm intervals. Image analysis was performed using the LAS AF software.

2.5. Cell cycle and apoptosis analysis

HeLa or A431 cells, seeded at the density of 2×10^5 cells/100 mm dish, were treated with 50 or 100 μg/ml of FQs for 24 h, then UVA irradiated at normoxic or hypoxic conditions, as above described. After 24 h, cells were trypsinized, washed in PBS and fixed in 70% ethanol for at least 2 h at −20 °C. Fixed cells were washed with PBS and resuspended in 1 ml of PBS containing 2 mg/ml RNase A, 0.05% NP-40, 5 μg/ml PI for at least 30 min at r.t. in the dark. Ten thousand cells were analyzed for each sample with a Coulter Epics XL flow cytometer. For detection of apoptosis, cells were plated at a density of 2×10^5 cells in 60 mm cell culture dishes. At the end of the treatments, adherent and floating cells were harvested and incubated for 10 min in Binding Buffer containing FITC-conjugated Annexin V (10 μl/10^6 cells) and, after washing, 20 μg/ml PI were added before flow cytometric analysis (Annexin V-FITC apoptosis kit, eBioscience Affymetrix Company). As an apoptosis positive control, Etoposide phosphate (VP-16) was used. Dual parameter cytograms of the red versus green fluorescence signals were obtained and at least 10,000 events were measured in the gate regions chosen for calculations.

2.6. Plasmid DNA damage

Irradiation was performed on samples containing 1 μg/μl of pGEX-T4 DNA and 50 μg/ml of each FQ in phosphate buffer (50 mM) at pH 7.4 for 30 min, at 37 °C and at physiological or hypoxic oxygen conditions [18]. The intensity of the irradiation was 12 mW/cm^2. The products were electrophoresed on a 0.8% agarose gel, and the corresponding bands were visualized by UV light. Gene Ruler 1-kb DNA Ladder (MBI Fermentas) was used as size marker. Digitalized images of the bands were acquired by a UMAX power