Compare two methods of measuring DNA damage induced by photogenotoxicity of fluoroquinolones

Ting ZHANG, Jun-ling LI, Jian XIN, Xiao-chao MA, Zeng-hong TU[1]

State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Shanghai Institute for Biological Sciences, Chinese Academy of Sciences, Shanghai 201203, China

KEY WORDS fluoroquinolones; phototoxic dermatitis; comet assay

ABSTRACT

AIM: To compare two methods of measuring DNA damage induced by photogenotoxicity of fluoroquinolones (FQ). **METHODS:** Lomefloxacin (LFLX), sparfloxacin (SPFX), ciprofloxacin (CPFX), and levofloxacin (LELX) were tested by comet assay and photodynamic DNA strand breaking activity under the different conditions of UVA irradiation. **RESULTS:** In comet assay, photogenotoxicity was evident at SPFX 1 mg/L, LFLX 5 mg/L, and CPFX 5 mg/L, and LELX 10 mg/L. In photodynamic DNA srand-breaking activity, SPFX and LFLX induced the conversion of the supercoiled form into the nicked relaxed form at 10-50 μmol/L, while CPFX at 25 μmol/L and LELX at 50 μmol/L. **CONCLUSION:** There were good correlations between the two methods to detect DNA damage induced by phototoxicity of fluoroquinolones. Photodynamic DNA strand breaking activity was a good method to detect DNA damage induced by photogenotoxicity of fluoroquinolones as well as comet assay.

INTRODUCTION

The fluoroquinolones (FQ) are employed very successfully in the treatment of abroad spectrum of infectious diseases. They act through inhibition of two type II DNA topoisomerase enzymes, DNA gyrase and topoisomerase IV. As a class, topoisomerases are essential in controlling the topological state of DNA by catalyzing supercoiling, relaxing, knotting, and catenation reactions which are vital for DNA replication, transcription, recombination, and repair. They have selective inhibition of bacterial topoisomerase enzymes relative to the human's[1]. However, even with this selectivity, the FQ as a class exhibit genotoxicity in a variety of *in vitro* and *in vivo* mammalian with the UVA irradiation[2].

It has been observed of experimental and clinical photogenotoxicity, photomutagenicity, and photocarcinogenicity associated with FQ exposure[3,4]. FQ have absorption peaks in the UVB (about 290 nm) and UVA (about 340 nm) region of the solar spectrum. Irradiation of cellular or *in vivo* systems at these wavelengths in the presence of these drugs can result in DNA strand damage, mutagenicity and tumorigenicity. It is reported that DNA strand damage was induced by reactive oxygen species (ROS), which may be important to the generation of photogenotoxicity[5].

The single cell gel electrophoresis assay (comet assay) is a rapid, simple, visual, and sensitive technique for measuring DNA damage in individual mammalian cells[6]. However, it needs fluorescence microscope, which is very expensive and resolution of fluorescence occurs under the irradiation of excitated wavelength.

[1] Correspondence to Prof Zeng-hong TU.
Phn 86-21-5080-5785. Fax 86-21-5080-7088.
E-mail zhtu@mail.shcnc.ac.cn
Received 2002-12-17 Accepted 2003-06-18

May we cannot take the photos at the exact time. Supercoiled circular DNA allows the detection of structural alteration such as strand break or damaged bases easily and is a very sensitive tool for damage detection. In fact, only one single-strand break is enough to trigger the conversion of the supercoiled form (form I) into the nicked relaxed form (form II); double-strand breaks can lead to the linear form (form III). The three forms are easily separated in agarose gel electrophoresis[7]. Supercoiled plasmid DNA pBR322 is commonly used in such experiments, but it is seldom used to detect the photogenotoxicity induced by FQ.

In our study, we used two methods to measure DNA damage induced by photogenotoxicity of FQ. Because FQ-induced skin phototo-xicity is assessed at several monochromator wavebands and found to be maximal at (365±30) nm which resemble the spectral output of the filtered PUVA source[8]. All experiments below were carried on under the condition of monochromatic irradiation at 365 nm.

MATERIALS AND METHODS

Chemicals Sparfloxacin (SPFX) was from Beite Pharmaceutical Co. Lomeflo-xacin (LFLX) was from Changzhou Pharmaceutical Co. Ciprofloxacin (CPFX) was from Shanghai Sanwei Pharmaceutical Co. Levofloxacin (LELX) was from Xinchang Pharmaceutical Co. Supercoiled plasmid pBR322 DNA was purchased fron Beijing Dinguo Biotech Co. All other chemicals were purchased from Shanghai Chemical Co. They were dissolved in phosphate buffer solution (PBS).

Cell culture Chinese hamster lung V79 cells were cultured in RPMI-1640 medium (Gibco-BRL, New York, USA) supplemented with glutamine 2 mmol/L, benzylpenicillin 100 kU/L, streptomycin 100 mg/L and 20 % newborn bovine serum. The cells were cultured in plastic T-50 flasks (Nunc, Roskilde, Denmark) at 37 ºC in a humidified atmosphere containing 5 % CO_2.

Irradiation of quinolone-treated cultures Single cell suspensions of 3×10^5 cells were plated in 35-mm diameter dishes (Corning, New York, USA) and following overnight incubation, medium was replaced with RPMI-1640 medium with or without FQ for 1 h at 37 ºC. Ice-cold PBS replaced this medium and cells were exposed on ice to 37.5 kJ/m^2 UVA irradiation. Intensity of UVA was measured at 365 nm by a UVX digital radiometer (Optical and Electrical Instrument Factory, BNU, China). Dishes were randomly placed under the source to compensate for any variations in intensity over the irradiation area. Foil-covered controls were dummy irradiation. Following irradiation, cells to be analyzed by the comet assay were detached from dishes with 0.25 % trypsin for 15 min at 4 ºC followed by gentle scraping.

Comet assay Frosted microscope slides were pretreated with 85 μL standard melting point agarose (1 %, w/v) solidified at 4 ºC under 18 mm×18 mm coverslip that was removed later. 10 μL cell suspensions was mixed with 75 μL of 1 % low melting point agarose and pipetted onto the lower agarose layer, covered with a coverslip and solidified at 4 ºC. Then the coverslip was replaced by 85 μL standard melting point agarose (1 %, w/v) solidified at 4 ºC under the coverslip that was later removed. Keeping the temperature at 4 ºC, slides were placed in lysis solution (sodium chloride 2.5 mol/L, edetic acid 100 mmol/L, Tris 10 mmol/L, and 10 % Triton X-100; pH =10) for 1 h and in denaturation buffer (edetic acid 1 mmol/L, sodium hydroxide 300 mmol/L; pH =12.5) for 40 min prior to electrophoresis for 30 min (25 V, 999 mA). Slides were washed three times (Tris/HCl 0.4 mol/L; pH 7.5) and stained with 80 μL of ethidium bromide 20 mg/L (EB). Slides were stored for up to 24 h in a humidified box in the dark at 4 ºC until being scored using a Leitz Diaplan fluorescence microscope. Fifty cells per coded slide were examined. Cells were classified into 5 categories: class 0: cells without any fluorescing particles; class 1: cells with only a few trailing fluorescing particles; class 2: cells with a thin trailing streak of particles; class 3: cells with strong comet; and class 4: comets with decomposed nucleus. Each experiment was repeated a minimum of three times and the mean SE comet score per 100 cells calculated. FQ in dark controls and UVA alone controls were included in each experiment[9,10].

Photodynamic DNA strand breaking activity FQ solution 5 μL (final concentration 10-50 μmol/L) in the dimethylsulfoxide (Me_2SO) and 9 μL of the PBS buffer were added to 6 μL of supercoiled plasmid pBR 322 DNA stock solutions in 1.5 mL Eppendorf tubes. The reaction mixtures were irradiated with a 70 KJ/m^2 UVA irradiation at room temperature. After irradiation, 5 μL of 0.1 % bromophenol blue in 30 % glycerol in TBE buffer (Tris 89 mmol/L, boric acid 89 mmol/L, edetic acid 2 mmol/L, pH 8.0) was added to the mixture. Electrophoresis was performed with 1.0 % agarose gel containing EB 0.5 mg/L. Aliquots 15 μL were loaded and eletrophoresed in 0.5×TBE buffer at 70 V for 2 h.

Then, the gels were photographed under irradiation with UV light (UVP dual-intensity transilluminator) through a red filter and scanned with FMBIO II muti-view to determine the quantity of the nicked relaxed form (form II) and the linear form (form III)[11,12]. Each experiment was repeated a minimum of three times and percentages of form II was calculated.

Statistics Data were expressed as mean±D. Statistical significance of differences between two groups was determined by *t*-test.

RESULTS

Comet assay FQ alone or UVA alone (37.5 kJ/m^2) produced only a low comet score (10-20) according to the 5 categories (0-400) and the vehicle did not enhance UVA-induced comet formation detected immediately after irradiation. The viability of cells at the highest concentration (10 mg/L) of FQ was ≥50 % detected by crystal violet. FQ showed a concentration-dependent photosensitization of comet formation attaining maximum mean comet scores of 130-320 (Fig 1). Photogenotoxicity was evident with SPFX 1 mg/L, with LFLX and CPFX 5 mg/L, with LELX 10 mg/L. The potency of DNA damage induced by FQ 10 mg/L was as the following order: SPFX>LFLX>CPFX>LELX.

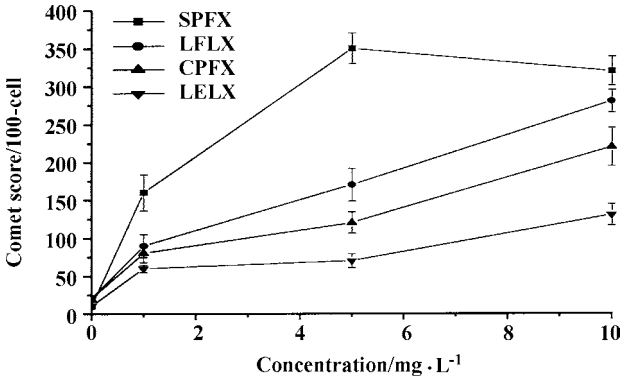

Fig 1. Comet scores produced in V79 cells immediately after exposure to 37.5 kJ/m^2 UVA in the presence of FQ. *n*=3. Mean±SD.

Photodynamic DNA strand breaking activity Exposures of supercoiled plasmid pBR322 DNA to FQ with 70 kJ/m^2 UVA irradiation can trigger the conversion of the supercoiled form (form I) into the nicked relaxed form (form II), when DNA damage to a severe degree, double-strand breaks can lead to the linear form (form III), which was observed at the highest concentration of SPFX (Fig 2). SPFX and LFLX induced the conversion of the supercoiled form into the nicked relaxed form at10-50 μmol/L, while CPFX at 25 μmol/L and LELX at 50 μmol/L. The DNA strand breaking activity was concentration-dependent. The potency of DNA strand breaking activity of FQ at 50 μmol/L was as the following order: SPFX>LFLX>CPFX>LELX. Direct DNA damage was assessed: the percentage of form II increased in a concentration-dependent manner in the presence of FQ (Fig 3).

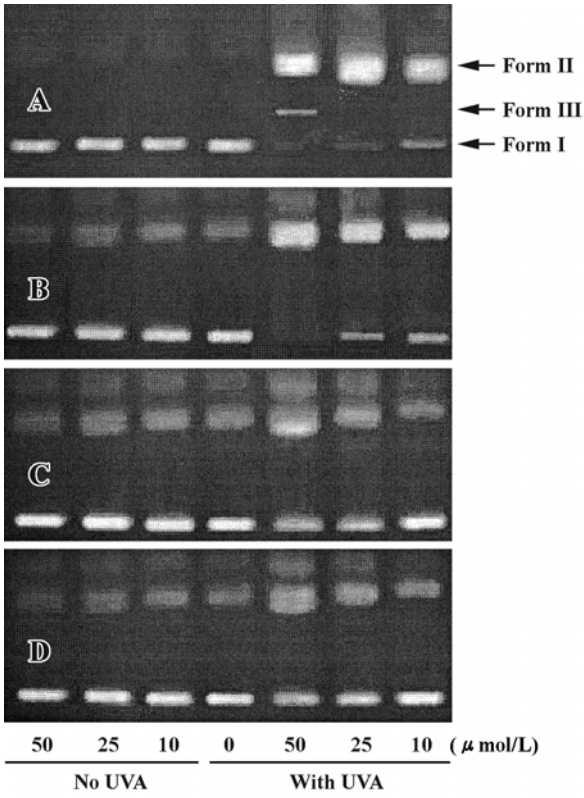

Fig 2. Photodynamic DNA strand-breaking activities of FQ. (A) SPFX; (B) LFLX; (C) CPFX; (D) LELX.

DISCUSSION

Many FQ have been reported to cause phototoxicity in clinical practice except moxifloxacin and gatifloxacin, which have methoxy group substitution at X8 position of FQ[13]. The extent of skin phototoxicity strongly differs from one FQ to another one. In clinical reports, SPFX and LFLX were shown to have relatively high phototoxic potential (6 %-10 %) compared with that of other FQ[1]. As the experiments we had done before, in Balb/c mice phototoxic test *in vivo*, SPFX and LFLX showed much higher phototoxic po-

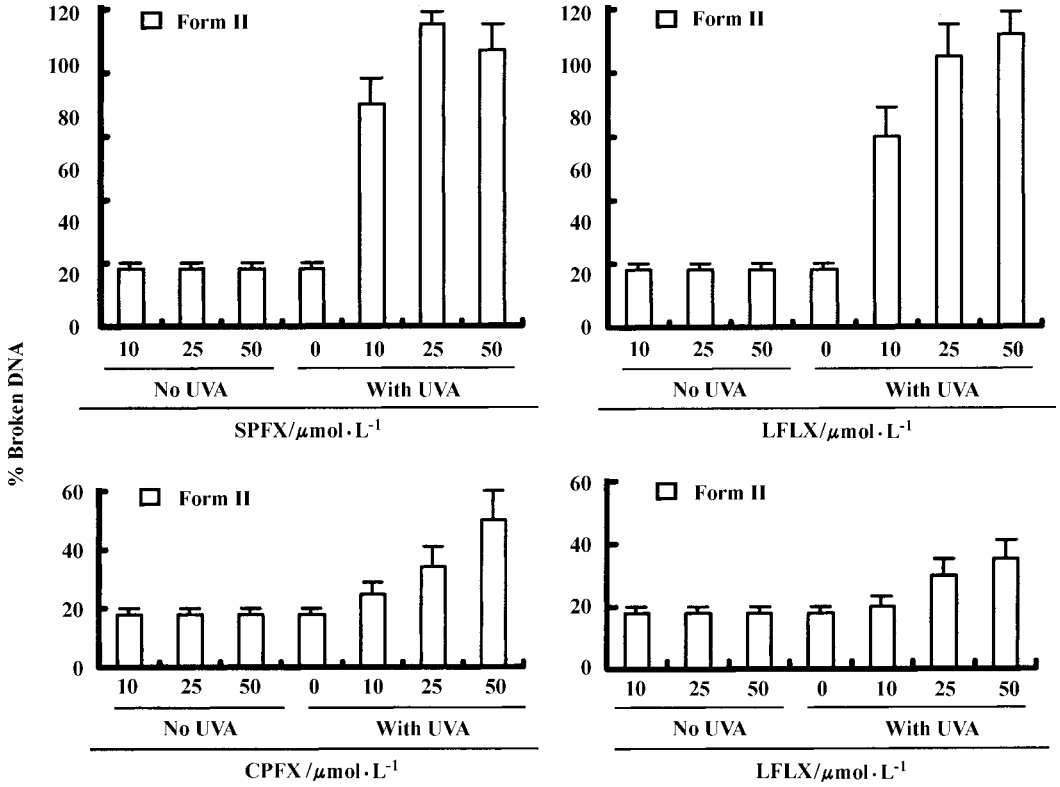

Fig 3. Electrophoretic patterns of pBR 322 plasmid DNA after UVA exposure in the presence of FQ. Percentages of form II was obtained from the densitometric analysis of the gel. *n*=3. Mean±SD.

tential than other FQ[14]. However, animal studies are expensive and time-consuming. The need for non-animal approaches in phototoxic testing is widely recognized. A number of *in vitro* models such as erythrocyte lysis, MTT or NRU cytotoxicity[15], micronucleus test[2], chromosomal aberration, comet assay[9], photodynamic DNA strand breaking activity, gene mutation, gene conversion, and ROS assay had been applied for evaluating the phototoxic potential of different kinds of FQ under the different UVA irradiation. However, till now, we cannot find some effective methods *in vitro* to detect phototoxic potential of FQ as standard principles. Comet assay is a traditional method to detect DNA damage since 1993. Photodynamic DNA strand breaking activity is also a good method to detect DNA damage and easy to operate. However, it was seldom used to detect DNA damage induced by photogenotoxicity of FQ before 1997. In this study, we compared them and ensured which was better.

In comet assay, DNA damage was evident with SPFX 1 mg/L, with LFLX and CPFX 5 mg/L, with LELX 10 mg/L. The potency of DNA damage induced by FQ 10 mg/L was as the following order: SPFX> LFLX>CPFX>LELX. Since SPFX and LFLX had been reported to show high phototoxicity in laboratory animals and humans, while CPFX and LELX were lower, the result of comet assay seemed to be related with the observation *in vivo*. In photodynamic DNA strand breaking activity, SPFX and LFLX induced the conversion of the supercoiled form into the nicked relaxed form at 10-50 μmol/L, while CPFX 25 μmol/L and LELX 50 μmol/L. When DNA damage to a severe degree, double-strand breaks can lead to the linear form, which was observed at SPFX 50 μmol/L. The DNA strand breaking activity was also concentration-dependent. The potency of DNA strand breaking activity of FQ 50 μmol/L was as the following order: SPFX>LFLX>CPFX>LELX. The result of photodynamic DNA strand breaking activity is also correlated with clinical reports and consistent with the data from comet assay.

It is reported that ROS participated in the mechanisms of cutaneous phototoxi-city induced by FQ. DNA damage in comet assay may be mainly due to formation of ROS generation in our study when FQ accumulated in the skin under the UVA irradiation. The ability of FQ

to photocleave plasmid DNA was reported to relate to binding affinity of FQ to DNA and ROS generated under UVA irradiation. So, maybe such two factors could influence photodynamic DNA strand breaking activity of FQ, which could explain the difference BAYy 3118 phtoproducts might tightly interact with DNA, while those of LFLX only induced DNA damage[12].

Among the methods to detect DNA damage induced by photogenotoxicity of FQ, comet assay was a good and sensitive method. However, the results from it could not fully explain phototoxic potential of FQ. As for photodynamic DNA strand breaking activity, the results from the consulted data except enoxacin may be consistent with clinical reports.

In conclusion, comet assay and photodynamic DNA strand breaking activity *in vitro* have good correlation to detect DNA damage induced by photogenotoxicity of FQ. The latter is easier to operate, seemed to be useful for predicting DNA damage induced by phototoxicity of FQ and also a good method for screening new FQ.

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