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DNA Damage and Cytotoxcity Induced in Mammalian Cells by a Tetramethylfuroquinolinone Derivative

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1,4,6,8-Tetramethyl-2H-furo[2,3-h]quinolin-2-one (FQ) is an angelicin isoster characterized by a strong photosensitizing activity. FQ shows a significant antiproliferative activity also in the dark, i.e., without UVA activation. The cytotoxic activity of FQ in the dark was detected in HeLa cells and in normal human lymphocytes; FQ showed notable antiproliferative effects, barely lower in comparison with ellipticine, used as a reference. Similar results were obtained studying the FQ's capacity for forming chromosome aberrations. For both FQ and ellipticine, the chromosomal damage correlated closely with cell killing; when compared with ellipticine at the same levels of survival, FQ appeared to be much less genotoxic. Using alkaline elution we have investigated the ability of FQ to damage DNA. The formation of equivalent amounts of single-strand breaks (SSB) and DNA-protein cross-links (DPC) was observed; in addition, these lesions appeared to be located at the same sites in DNA. Experiments carried out with neutral elution demonstrated the formation of double-strand breaks (DSB). All these data are consistent with an inhibition of topoisomerase II; this hypothesis was confirmed performing an enzymatic test in vitro using topoisomerase II from *Drosophila melanogaster* embryos. Environ. Mol. Mutagen. 29:256–264, 1997.

Key words: furoquinolinones; topoisomerase II; DNA-protein cross-links; double-strand breaks; chromosomal aberrations; single-strand breaks

INTRODUCTION

Furocoumarins are active photosensitizing drugs widely employed in photochemotherapy (psoralens plus UVA [PUVA]) [Parrish et al., 1982], in research on the structure of various biological macromolecules [Cimino et al., 1985] and on DNA repair [Van Houten, 1990]. Their activity is because of the damage induced by UVA-activated drug in different molecules inside cells, but the main interaction seems to take place with DNA [Ben-Hur and Song, 1984; Averbeck, 1984]. After UVA irradiation, furocoumarins interact with pyrimidine bases of DNA, forming monofunctional and bifunctional adducts. The bifunctional lesions (interstrand cross-links [ISC]) seem to be related to the severe biological effects of furocoumarins, such as cytotoxicity and mutagenicity and increased risk for cancer [Rodighiero, 1978]. To reduce these undesired biological effects, various monofunctional furocoumarins have been synthesized and studied [Bordin et al., 1991, 1992].

Following this research line, we have studied some angular furoquinolinones, which could be regarded as angelicin isosteres [Rodighiero et al., 1996]; it is known that, for geometrical reasons, angelicins can hardly form ISCs [Bordin et al., 1991]. One of these derivatives, 1,4,6,8-tetramethyl-2H-furo[2,3-h]quinolin-2-one (FQ), is characterised by a strong photosensitizing activity [Bordin et al., 1996]. Now we have also observed that this compound is capable of inducing a significant antiproliferative activity in mammalian cells without UVA activation. This behaviour is very similar to that observed with some benzopsoralen derivatives having a fourth ring condensed at the furan side, which were prepared with the aim to obtain photoactive monofunctional drugs [Bordin et al., 1992]. In fact, some of these benzopsoralens showed a marked antiproliferative activity also in the dark. We were able to demonstrate that this activity was related to the inhibition of topoisomerase II [Pani et al., 1994]. In this paper we describe some cytotoxic and genotoxic features of FQ; in particular, we evaluated the relationship between the antiproliferative activity, the production of chromosomal aberrations, and the damage induced in DNA by incubation in the dark in normal human lymphocytes and HeLa cells. As determined by alkaline and

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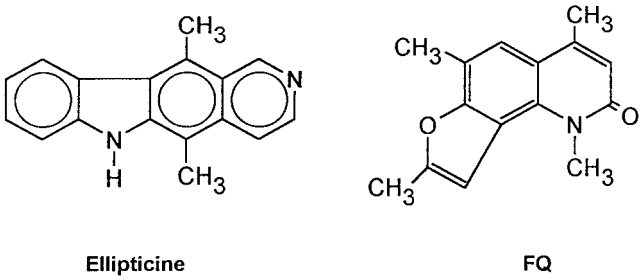

Fig. 1. Molecular structure of the compounds studied. FQ: 1,4,6,8-tetramethyl-2H-furo[2,3-h]quinolin-2-one; Ellipticine (5,11-dimethyl-6H-pyrido[4,3-b]carbazole) was used as a reference.

neutral elution, FQ was able to provoke DNA damage that was consistent with topoisomerase II inhibition. In addition, we evaluated the ability of FQ to inhibit the activity of mammalian topoisomerase II in vitro.

MATERIALS AND METHODS

Chemicals

FQ [Rodighiero et al., 1996] was prepared by chemical synthesis. Ellipticine, used as a reference, was obtained from Sigma Chemical Co. (St Louis, MO). The molecular structures of these compounds are reported in Fig. 1.

Compounds were dissolved in dimethyl sulfoxide (DMSO) (4.5 mM) and the solutions were stored frozen in plastic tubes. Just before the experiments, a calculated amount of the drug solution was added to phosphate-buffered saline (PBS) or to the growth medium to a final solvent concentration of 0.5%. ^{3}H-thymidine (4.77 Tbq/mM) and ^{14}C-thymidine (2.2 GBq/mM) were obtained from Amersham International, Inc. (Amersham, U.K.). Proteinase K was obtained from Boehringer Mannheim GmBH (Mannheim, Germany). Tetrapropylammonium hydroxide, 1 M aqueous solution, was purchased from Sigma Chemical Co.

Cell Culture

HeLa cells (kindly provided by Prof. F. Majone, Dept. of Biology of Padua University, Italy) were grown as monolayers in Nutrient Mixture F12 Ham medium (Sigma Chemical Co.) supplemented with 10% fetal calf serum (Biological Industries, Kibbutz Beth Haemek, Israel) and the antibiotics penicillin (50 units/mL) and streptomycin (50 μg/mL). Trypsin (0.25%, Boehringer Mannheim) was routinely used for subculture.

Whole blood (0.5 mL), collected from healthy adult female donors using standard venipuncture methods, was incubated in RPMI 1640 medium (Sigma Chemical Co.; 4.5 mL), supplemented with 10% fetal calf serum, 50 units/mL penicillin, and 50 μg/mL streptomycin. Cell division was promoted by the addition of 1% phytohemagglutinin (GIBCO BRL, Paris). The cultures were set up in duplicate and incubated for 72 hr in a humidified 5% CO_2 atmosphere at 37°C. All experiments were carried out under controlled red light.

Chromosome Preparations

The method used to assay chromosome aberrations has been reported previously [Scott et al., 1990]. Briefly, suspensions of 2×10^6 HeLa cells/mL in log-phase cultures were diluted with growth medium in 100-mm Petri dishes (Falcon Labware, Oxnard, CA). Each dish received about 8×10^5 cells in 10 mL of medium, which was changed 24 hr after seeding. Compounds to be studied were added to the cells for 3 hr in prewarmed complete growth medium; drugs were removed by washing the cells twice with 10 mL of PBS. For human lymphocytes, the treatment with tested compounds was made at the 48th hr after seeding. Cells were then incubated for 24 hr at 37°C. Colchicine (0.4 μg/mL; Merck, Darmstadt, Germany) was added before fixing preparations during the last 4 hr for HeLa cells, and during the last 2 hr for lymphocytes. Metaphase cells were dislodged by gently pipetting the overlaying medium and collected by centrifuging the suspensions at 1,000 rpm for 8 min. The cell pellet was suspended in 5 mL hypotonic buffer (0.075 M KCl) at 37°C for 12 min for lymphocytes, and for 20 min for HeLa cells; cells were then fixed in cold methanol-acetic acid (3:1). Slides were stained in 5% Giemsa.

Clonogenic Survival

Trypsinized HeLa cells were grown at a density of 200 cells into a 60-mm dish. Triplicate cultures were established for each treatment. After 24 hr, the medium was removed and replaced with fresh medium containing the compound to be studied at the appropriate concentrations; cells were incubated for 3 hr at 37°C in the dark in a 5% carbon dioxide atmosphere. The dishes were then washed with PBS and then 200 cells from each treated and untreated culture were seeded in complete growth medium. After 7 days of incubation, colonies were stained and counted, discarding colonies with less than 50 cells. The efficiency of the clonal growth, that is, the ratio between the number of colonies formed and the number of cells seeded, was then calculated and used to normalize the cytotoxicity induced by the drugs.

Nonclonogenic Assay

Cytotoxicity was studied in human lymphocytes using the trypan blue dye exclusion test [Durkin et al., 1979]. Cells at a concentration of 2×10^5/mL were incubated for 3 hr in the presence of different concentrations of the compounds to be tested. Cells were then incubated for 4 min with 0.25% trypan blue (Sigma Chemical Co.) and 5% fetal calf serum. Viable cells were identified by their ability to exclude dye, whereas the dye diffuses into nonviable cells. At least 100 cells were counted for each experimental point recorded.

Detection of DNA Damage

DNA damage was detected by alkaline and neutral elution. Alkaline elution was carried out according to Kohn [1991]; each experiment was carried out using an internal standard, i.e., untreated cells labelled with ^{3}H-thymidine, while treated cells were labelled with ^{14}C-thymidine.

HeLa cells in exponential growth were labelled by overnight incubation in the presence of ^{3}H-thymidine (7.4 KBq/mL) or ^{14}C-thymidine (3.7 KBq/mL). The radioactive medium was removed and replaced by fresh growth medium containing the compound to be studied for ^{14}C-cells and containing 0.5% DMSO for ^{3}H-cells; in both cases, the cells were incubated for 3 hr in the dark. The cells were then washed with PBS.

In the determination of single-strand breaks (SSB), about $0.5-1.0 \times 10^6$ treated ^{14}C-cells were mixed with equal amounts of ^{3}H-cells that had been exposed to 6 Gy of gamma rays; the mixture was deposited on a polycarbonate filter (pores 2 μm in diameter, Nucleopore Corp., Pleasanton, CA) in a Swinnex-25 filter holder (Millipore Corp., Bedford, MA) and immediately lysed with 2% sodium dodecylsulfate (SDS), 0.1 M glycine, 0.025 M Na_2 ethylenediaminetetraacetic acid (EDTA), pH 10, (5 mL); thereafter, the solution was allowed to flow through by gravity. Then 2 mL of the same solution containing 0.5 mg/mL of proteinase K was gently poured on the filter, followed by 40 mL of the eluting solution (tetrapropylammonium hydroxide-EDTA-0.1% SDS,

pH 12.1). The elution was carried out by a Gilson Minipulse peristaltic pump, at a flow of 0.03–0.04 mL/min. The fractions were collected with a Gilson fraction collector (approximately 3.5 mL/fraction) and the radioactivity of both isotopes in each fraction was then determined.

To detect DNA-protein cross-links (DPC), about $0.5–1.0 \times 10^6$ treated ^{14}C-cells were mixed with an equal amount of standard ^{3}H-cells, the cell suspension was cooled in ice, and exposed to 30 Gy of gamma rays. Elutions were then performed as described above for SSB determinations, but polyvinyl chloride filters (pores 5 μm in diameter) (Nucleopore Corp.), instead of polycarbonate ones, were employed, and the treatment with proteinase K was omitted.

Protein-associated strand breaks (PASB) were detected in conditions similar to those used for DPC assays, but the samples were not subjected to gamma irradiation. Thus, if SSB and DPC were distributed independently of each other, some of the single-strand pieces of DNA generated by SSB induction could have a chance to be free of linked protein and should elute at an increased rate compared with the no-drug controls. On the contrary, if there is no detectable increase in the elution rate in the drug-treated cells, we can conclude DPC must be associated with SSB.

Double-strand breaks (DSB) were detected by neutral elution, which differs from the alkaline elution by the pH of the eluting solution (9.5 instead of 12.1). In addition, the internal standard was omitted. In fact, in alkaline elution, DNA elutes as single-stranded molecules, whereas the elution of double-stranded DNA strongly depends upon the number of the cells lysed on the filter [Kohn 1991], so that the cell number must be carefully controlled. All the experimental conditions were the same as described above for SSB.

Calculations

Calculations were performed according to Kohn [1991]; SSB were determined according to the following equation:

$$p - p_0 = \frac{K - K_0}{K_1 - K_0} \cdot p_{bR}$$

where p and p_0 are the SSB frequencies observed with the drug and in the untreated control, K, K_0, and K_1 are the slopes of the elution profiles obtained with the drug, the gamma rays, and observed in the untreated cells, respectively. The p_{bR} is the SSB frequency generated by the gamma rays (6 Gy).

The DPC number was calculated using the PASB method;

$$p = p_{bR} \cdot \left[\frac{1}{1 - r} - \frac{1}{1 - r_0} \right]$$

p_{bR} is the SSB frequency induced by the gamma rays (30 Gy), p is the DPC frequency induced by the drug, and r and r_0 are the fractions of DNA retained on the filter in the treated and untreated cells, respectively.

The results obtained with the neutral elution assay were expressed as K values according to the formula [Noviello et al., 1994]:

$$K = \frac{V}{-\ln(r)}$$

where K is the average elution rate constant (ln/mL) of DNA, r is the fraction of DNA retained on filter, and V is the eluted volume. The formula reflects the assumption of first-order kinetics for DNA elution, as a first approximation [Kohn, 1991].

Gamma ray exposures were always performed on ice using a ^{60}Co source working at the Reparto Applicazioni, Legnaro, Padova, Istituto di Fotochimica e Radiazioni d'Alta Energia, FRAE, C.N.R., with a dose-rate of 1.7 Gy/min, as determined by Fricke solution.

Radiochemical Determinations

The radioactivity measurements were performed using Instagel (Packard Instruments, Meriden, CT) as a scintillation fluid. All determinations were carried out by a Packard A 300 CD spectrometer. Double-isotope counting was accomplished automatically on the bases of quenching curves obtained using ^{3}H-and ^{14}C-radioactivity standards.

Inhibition of Topoisomerase II Activity

The inhibition of topoisomerase II activity was studied using a purified enzyme from *Drosophila melanogaster* embryos (USB, from Amersham Italia S.r.l.) [Tao-Shih Hsieh, 1983]. PM2 DNA, 0.125 μg (from Böhringer Mannheim), was incubated for 15 min at 30°C in the presence of 2 units topoisomerase II (1 unit is defined as the activity capable of relaxing 0.3 μg of super-coiled DNA) in the presence of reaction buffer (10 mM Tris-HCl, pH 7.9; 50 mM NaCl; 50 mM KCl; 5 mM $MgCl_2$; 0.1 mM EDTA); 15 μg/mL bovine serum albumin [BSA]; 1 mM adenosine 5′-triphosphate [ATP]). Aliquots of 2 μL of an FQ solution in DMSO (4.5 μM) were added to reach the following final concentrations: 1, 10, 20, 40, 160 μM. A suitable amount of reaction buffer was then added to every sample to reach the final volume of 20 μL. The reaction was blocked by adding 7 mM EDTA (3 μL) containing 0.77% of SDS. Two microliters of bromophenol blue containing 15% glycerol was added to the samples and the electrophoresis was carried out on 0.7% agarose gel containing 40 mM Tris-acetate, pH 8.2, 1 mM EDTA (TAE) for 90 min. The gel was stained for 1 hr in aqueous ethidium bromide (0.5 μg/mL) and then photographed by a Polaroid camera.

RESULTS

Detection of Cytotoxicity

The dye exclusion test was used to evaluate the cytotoxicity induced in normal human lymphocytes after 3 hr of incubation in the presence of different concentrations of FQ. Figure 2A shows the data thus obtained; FQ induced an evident cytotoxic effect, even if it appeared to be lower in comparison with ellipticine, which was used as a reference.

The clonogenic assay was used to determine survival of HeLa cells after treatment (3 hr) with increasing concentrations of FQ (Fig. 2B), with ellipticine used as a reference. The results seem to be similar to those obtained with lymphocytes; actually, both compounds induced an evident inhibition of the colony-forming ability of HeLa cells.

Analysis of metaphase cells showed that FQ was able to induce chromosomal aberrations in HeLa cells (Fig. 2C). Even in these experiments, ellipticine appeared to be more active. However, in this case the difference between the activity of the two compounds is more pronounced. In fact, contrary to ellipticine, the chromosomal aberrations induced by FQ rapidly formed a plateau with a small increase even when the drug concentration was doubled.

A detailed analysis of chromosomal damage carried out in HeLa cells and in normal human lymphocytes

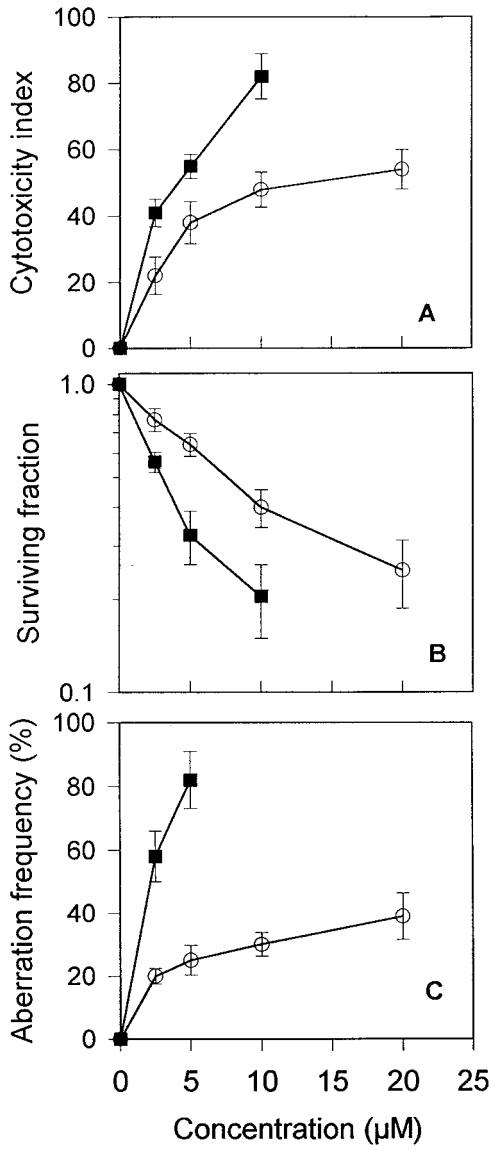

Fig. 2. Cytotoxic effects of FQ. **A:** Cell viability was determined by trypan blue dye exclusion in normal human lymphocytes (1×10^6/mL) after 24 hr of incubation in the presence of increasing concentrations of FQ (○) and of ellipticine (■). **B:** Clonal growth capacity of HeLa cells cultivated in vitro after 3 hr of incubation in the presence of different concentrations of FQ (○) and of ellipticine (■). **C:** Chromosomal aberrations induced by FQ (○) and ellipticine (■) in HeLa cells after 3 hr of incubation in the presence of different concentrations of the compounds. Ellipticine was used as a reference. The bars represent the standard errors for at least three independent determinations.

showed that FQ induced chromatid breaks, interchanges, and gaps; dicentric chromosomes, isochromatid breaks, chromosome, and chromatid rings were also present (Table I). No significant differences in the types of aberrations induced by FQ and ellitpicine were observed. FQ also produced a high percentage of polyploid cells (data not shown).

Detection of DNA Damage

Strand Breaks

The induction of SSB in DNA of HeLa cells was studied by alkaline elution after incubation (3 hr) in growth medium in the presence of FQ (20 μM). The results are shown in Fig. 3A. DNA from treated cells eluted faster than that from untreated controls, suggesting that FQ is capable of inducing SSB in DNA.

Figure 3B shows the data obtained studying the formation of DSB by neutral elution. In these experimental conditions DNA from treated cells eluted faster; therefore, it is clear that FQ can induce DSB into DNA. The damage is particularly evident when the FQ concentration is increased to 40 μM. Ellipticine, used as a reference, was able to induce DNA DSB in a drug concentration-dependent manner, but clearly it appeared to be much more effective than FQ.

DPC

In Fig. 4A, the data related to DPC detection in HeLa cells are reported. Three hours of incubation in the presence of FQ (20 μM) lead to a marked retention of DNA on the filter, suggesting the formation of a remarkable amount of DPC. Figure 4B shows the data obtained studying the formation of PASB; these experiments were carried out using the usual method for DPC detection, but omitting the irradiation with gamma rays. The elution profile of DNA from cells exposed to FQ was superimposable to that obtained in untreated cells; this means that there are no DNA fragments free of DPC, a classical picture of the formation of PASB by topoisomerases. Nevertheless, the induction of SSB can always be detected by exposure to gamma rays, as demonstrated by exposing an untreated sample to 5 Gy of gamma rays. Hence, the SSB produced during incubation with FQ were specifically hidden by the DPC, because these two lesions are associated, while SSB randomly induced by gamma rays were not masked.

The relative frequencies of DPC and SSB induced by treatment with FQ are summarized in Table II. From these data, it appeared that they are very similar. In fact, the frequency of DPC was within a factor of 2 with respect to the frequency of SSB, a ratio that, according to Kohn [1991], can be considered within the experimental error. Therefore, we can conclude that all the single-strand DNA breaks induced by FQ are protein associated.

Inhibition of Topoisomerase II Activity

The FQ ability to inhibit the topoisomerase II activity was studied by means of relaxation assay of supercoiled DNA of PM2 phage by electrophoresis on agarose gel; the data obtained at different FQ concentrations, from 1 to 160 μM, are reported in Fig. 5. Lane 1 shows the

TABLE I. Chromosomal Aberrations Induced in Human Lymphocytes and HeLa Cells Exposed to the Tested Compounds

Cells tested	Conc. (μM)	Aberrant metaphases	Chromosome aberrations		Chromatid aberrations					Total aberrations (%)
			Dic.	Rings	Gaps	Breaks	Rings	Interchanges	Isoc. breaks	
Lymphocytes										
Controls	0	4	—	—	—	—	1	2	1	4
FQ	5	24	4	2	4	6	1	7	1	25
Ellipticine	5	53	8	2	12	19	2	23	6	72
HeLa cells										
Controls	0	6	1	2	1	1	—	—	1	6
FQ	5	21	3	3	4	4	1	9	1	25
	10	26	4	1	5	9	1	8	2	30
	20	34	5	2	5	11	2	12	2	39
Ellipticine	5	54	14	4	9	16	2	32	3	80

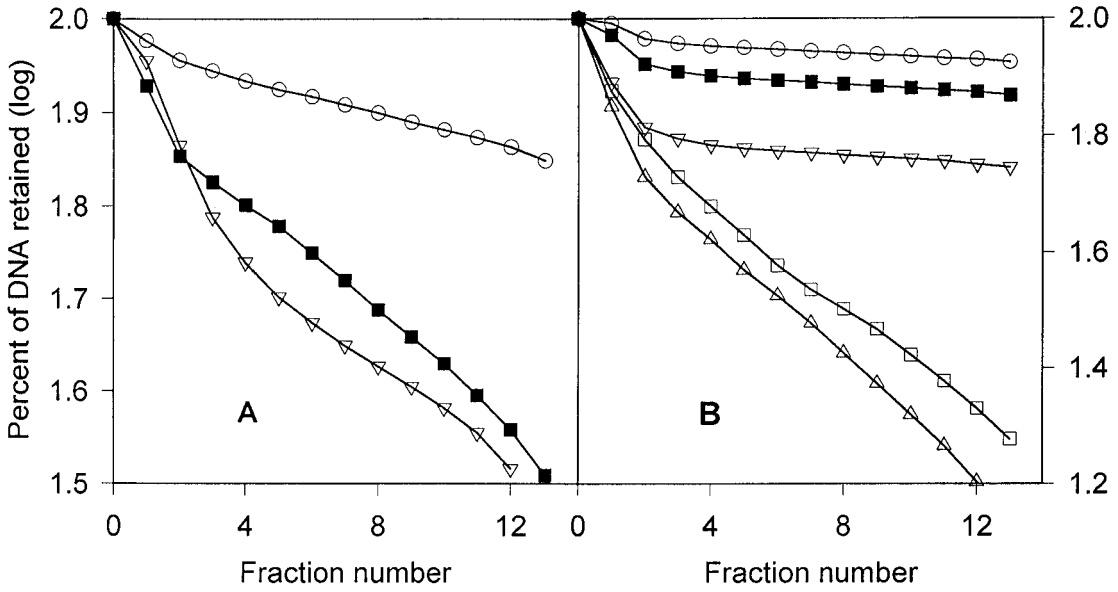

Fig. 3. Detection of strand breaks in DNA of HeLa cells by alkaline and neutral elution. **A:** SSB formation after incubation (3 hr) in the presence of FQ (20 μM) detected by alkaline elution. The symbols are untreated control cells (○), cells submitted to 6 Gy of gamma rays only (▽), cells incubated with FQ (■). **B:** DSB formation after incubation (3 hr) in the presence of FQ or ellipticine detected by neutral elution. The symbols are untreated control cells (○); cells incubated with FQ, 20 μM (■) and 40 μM (▽); cells incubated with ellipticine, 5 μM (□) and 10 μM (△).

electrophoretic pattern of PM2 DNA, where the two bands correspond to the respectively relaxed (slower) and supercoiled (faster) forms. Lane 2 shows the effect of the topoisomerase II activity, where the supercoiled form completely disappeared. Lanes 3–7 show the inhibition of topoisomerase II induced by FQ: at the highest concentration of the drug (lane 3) the band corresponding to the supercoiled form of PM2 DNA is comparable to that of the control sample (lane 1), decreasing (lanes 4–7) with the FQ concentration, thus demonstrating a concentration-dependent inhibition of topoisomerase II activity induced by the drug. Lane ''e'' shows the electrophoretic pattern obtained in an analogous experiment with ellipticine, used as a reference; its behaviour is very similar to that shown by FQ.

DISCUSSION

FQ is a photosensitizer capable of inducing strong modifications into mammalian cell DNA and, as a consequence, marked antiproliferative effects [Bordin et al., 1996]. Because of these features, it was proposed as a

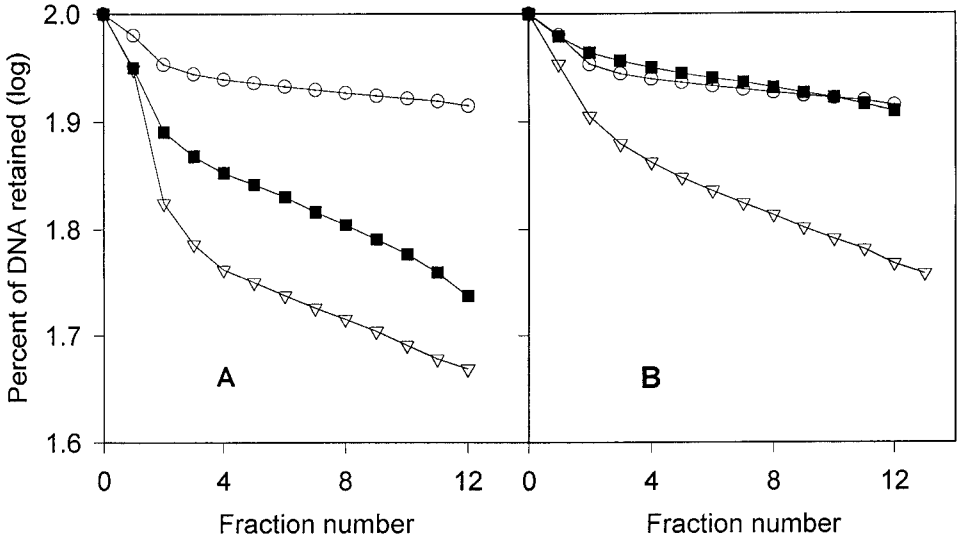

Fig. 4. Detection of DPC in HeLa cells by alkaline elution. **A:** Formation of DPC by incubation (3 hr) in the presence of FQ (20 μM); after the treatment, the cells were submitted to alkaline elution on PVC filters and in the presence of proteinase K. The symbols are untreated control cells (○), cells submitted to 30 Gy of gamma rays only (▽), and cells submitted to 30 Gy of gamma rays and incubated with FQ (■). **B:** Formation of DPC associated with SSB by incubation (3 hr) in the presence of FQ (20 μM); the cells were then submitted to alkaline elution on PVC filters in the absence of proteinase K. The symbols are untreated control cells (○), cells submitted to 5 Gy of gamma rays (▽), and cells incubated in the presence of FQ (20 μM) (■).

TABLE II. DNA Damage Determined by Alkaline Elution

FQ concentration (μM)	Lesions induced per million nucleotides		
	SSB ± D.S.	DPC ± D.S.	Ratio
20	0.489 ± 0.06	0.907 ± 0.15	1.8
40	0.995 ± 0.043	1.23 ± 0.113	1.23

new drug for PUVA photochemotherapy and photoferesis [Rodighiero et al., 1996; Bordin et al., 1996]. In this paper the activity of FQ in the dark, i.e., without UVA activation, in mammalian cells was investigated. This aspect is very important in view of its possible use in PUVA therapy. We have investigated this problem using HeLa cells and normal human peripheral lymphocytes. We chose ellipticine as a reference compound [Auclair, 1987; Liu, 1989].

In the studies on the vital trypan blue exclusion and on the cloning efficiency, FQ appeared to be capable of inducing clear cytotoxic effects, even if barely lower in comparison with ellipticine. Using probit analysis, we calculated the lethal doses at 50% for the clonogenic assay; they are 7.4 and 3.1 μM for FQ and ellipticine, respectively. Performing a comparison by probit analysis between the two data sets, we obtained two parallel straight lines, as confirmed by the χ^2 test (data not shown). Similar results were obtained comparing the data related to the dye exclusion. However, submitting to the same analysis the data related to the induction of chromosome aberrations, we obtained two straight lines having very different slopes. FQ induces a lower number of chromosome aberrations in comparison with ellipticine, even if no remarkable qualitative differences can be observed in the types of aberrations induced both in lymphocytes and in HeLa cells.

These results suggest that the two compounds induce antiproliferative effects by similar mechanisms, whereas there are some differences in the mechanism of formation of chromosome damage by the two drugs.

This difference is more evident if we consider the data reported in Fig. 6, in which we have plotted the aberration frequency against the surviving fraction in HeLa cells. It is evident that, at the same level of survival, FQ and ellipticine induce very different amounts of chromosome aberrations. The same picture was obtained by plotting the aberration frequency against the cytotoxic index observed in human lymphocytes (see again Fig. 6). On the basis of these data, we can suppose that FQ induces antiproliferative effects that are associated with a reduced genotoxicity in comparison with ellipticine. At present we cannot explain this different behaviour, which requires it to be further investigated. In fact, as described before, both compounds induce practically the same lesions; we could suppose there are some differences in their repair, as already observed for the lesions induced into DNA by ellipticine itself, and *N*-2-(diethylaminoethyl)-9-hydroxyellipticinium chloride [Djuric et al., 1992].

The hypothesis concerning an analogous mechanism of action for FQ and ellipticine was supported by the data obtained studying the damage induced in DNA in vivo.

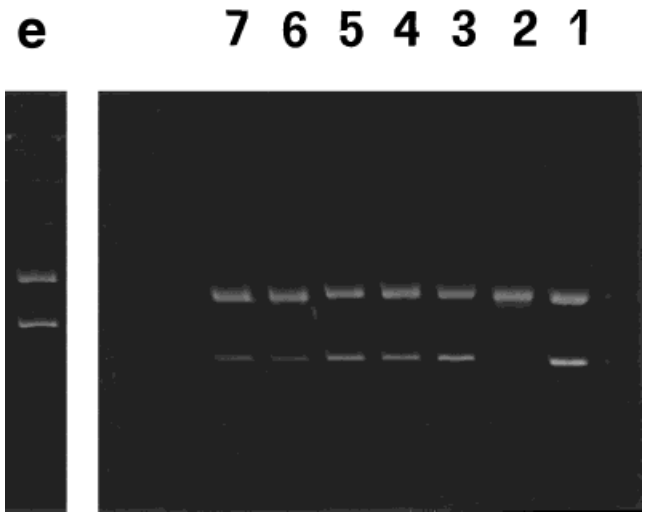

Fig. 5. Inhibition of topoisomerase II activity studied by the relaxation assay. PM2 DNA was incubated in the presence of topoisomerase II and of different FQ concentrations. Agarose gel electrophoresis of the samples is shown. **Lane 1:** PM2 DNA alone, which migrates as two bands. **Lane 2:** PM2 DNA incubated in the presence of topoisomerase II, showing only the relaxed form (the slower band). **Lanes 3–7:** PM2 DNA incubated in the presence of topoisomerase II and of decreasing FQ concentrations: 160, 40, 20, 10, 1 μM, respectively. **Lane "e":** PM2 DNA incubated in the presence of topoisomerase II and ellipticine (160 μM) assayed in an analogous experiment.

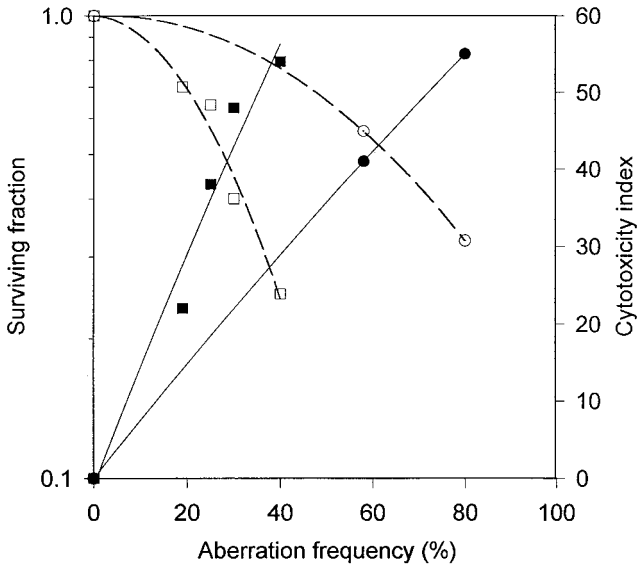

Fig. 6. Relationship between aberration frequency and clonogenic survival in HeLa cells (dotted lines, Y axis on the left). Relationship between aberration frequency and cytotoxic index in normal human lymphocytes (solid lines, Y axis on the right). The symbols are for clonogenic survival: FQ (□), ellipticine (○); for cytotoxic index: FQ (■), ellipticine (●). The values are taken from the data reported in Fig. 2.

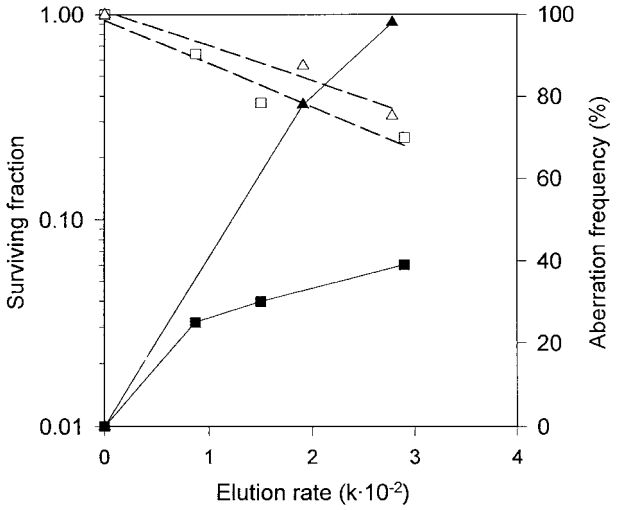

Fig. 7. Relationship between the formation of DSB (expressed as elution rate) and clonogenic survival in HeLa cells (dotted lines, Y axis on the left). Relationship between elution rate and aberration frequency in HeLa cells (solid lines, Y axis on the right). The symbols are for clonogenic survival: FQ (□), ellipticine (△); for aberration frequency: FQ (■), ellipticine (▲). The values are taken from the data reported in Figs. 2 and 3.

In fact, using alkaline elution, we observed that FQ is able to form SSB and covalent DPC to an equivalent extent. It is known that this picture is characteristic for the inhibitors of topoisomerase activity. The ratio between SSB and DPC was within the factor of 2, which, according to Kohn [1991], is consistent with an equivalence between these two lesions. This picture is typical for inhibitors of both topoisomerase I and topoisomerase II; as it is known, we can distinguish between these two classes of inhibitors, simply observing if the compound induces SSB or DSB, which are formed by topoisomerase I and topoisomerase II, respectively [Kohn, 1991]. Using the neutral elution, we observed that FQ induces DSB into DNA. Moreover, using a modified method of alkaline elution, we were able to demonstrate that SSB and DPC induced by FQ are located at the same site, thus confirming PASB are formed.

Therefore, these results are consistent with the hypothesis that FQ inhibits topoisomerase II. In fact, performing some experiments in vitro with topoisomerase II from embryos of *Drosophila melanogaster*, we observed that FQ inhibits the activity of this enzyme even at very low concentrations. We have already observed that FQ forms a molecular complex with DNA by intercalation into base pairs [Rodighiero et al., 1996]; we can therefore suppose that it forms a ternary complex with the enzyme and DNA, thus stabilizing strand breaks and covalent binding of DNA to protein (cleavable complex).

In Fig. 7 we plotted the elution rate observed in DSB detection against the survival obtained in the clonogenic assay. We can observe an evident relationship between the damage induced in DNA and the inhibition of the clonal growth: FQ and ellipticine generated two superim-

posable regression lines. Therefore, we can think that the lesions induced by the two compounds have the same cytotoxicity. The picture appears very different when the number of DSB is plotted against the frequency of chromosomal aberrations (see again Fig. 7). In this case, the lesions induced by ellipticine seem to be more effective in producing chromosomal damage than those induced by FQ. At present, it is impossible to explain adequately this result. As we have seen, the trapping of the ''cleavable complex'' by topoisomerase inhibitors produces both SSB and DSB; DSB are usually regarded as the ultimate lesions leading to chromosomal damage [Palitti, 1993], which must be considered the main cause of cell death.

Ellipticine is a very good intercalator [Kohn at al., 1975], capable of binding strongly to DNA. This interaction was classified as a pure intercalative process, with a unique class of intercalative site [Schawaller et al., 1990]. FQ forms a weaker molecular complex with DNA, with an association constant about two orders of magnitude lower in comparison with ellipticine [Rodighiero et al., 1996]. Thus, we have evidence that FQ forms an intercalative complex, but at present we cannot exclude that other types of interaction between FQ and DNA have taken place at the same time, e.g., by an outside binding, as described for some ellipticine derivatives having weak intercalative properties [Monnot et al., 1991]. These possible differences in the interaction modes with DNA might be taken into account to explain the biological effects induced by FQ and ellipticine, in particular, their different ability to induce chromosomal aberrations. Another possible explanation for their behaviour could be a different ability of these two drugs to act at different steps of the cell cycle, a property observed with ellipticine by Sakamoto-Hojo et al. [1988] and Sakamoto-Hojo and Takahashi [1991] using human lymphocytes. At present we have insufficient data for FQ; therefore, this point requires further investigations in order to be clarified.

All the data concerning FQ's activity are very similar to that already obtained from studying the activity of benzopsoralens in mammalian cells. These derivatives are active photosensitizing compounds but are also capable of inducing antiproliferative and genotoxic effects by simple incubation in the dark [Bordin et al., 1992; Pani et al., 1994]; they produce chromosomal aberrations together with a marked delay in the progression of the mitotic cycle. Studying the damage induced in DNA by benzopsoralens and their effect on topoisomerase II in vitro, we concluded that they inhibit this enzyme. Thus, with the present study we add further evidence on the capacity of furocoumarin derivatives to inhibit topoisomerases and to induce antiproliferative effects by simple incubation in the dark. In fact, we demonstrated that FQ, in addition to its strong photosensitizing activity, is capable of inducing evident cytotoxic and genotoxic effects even without UVA activation, and both these activities could be related to its ability to inhibit topoisomerase II activity. Therefore, this property must be taken into account for its possible use in PUVA photochemotherapy.

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