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Photobiological properties of 1-(3′-hydroxypropyl)-4,6,8-trimethylfuro[2,3-*h*]quinolin-2(1*H*)-one, a new furocoumarin analogue

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Abstract

A new furoquinolinone derivative, 1-(3′-hydroxypropyl)-4,6,8-trimethylfuro[2,3-*h*]quinolin-2(1*H*)-one (HPFQ, **4**), was prepared, in which the nitrogen atom in position 1 carries a hydroxypropyl chain. The antiproliferative activity of HPFQ was studied in comparison with its analogue 1,4,6,8-tetramethylfuro[2,3-*h*]quinolin-2(1*H*)-one (FQ) and 8-methoxypsoralen (8-MOP). By incubation in the dark, HPFQ, although retaining antitopoisomerase II activity, appeared less effective than FQ. Upon UVA irradiation, HPFQ produced little amounts of singlet oxygen, but detectable levels of superoxide anion; like FQ, HPFQ induced numbers of DNA–protein cross-links, but no interstrand cross-links in mammalian cells. The HPFQ phototoxicity was comparable to that of FQ and 8-MOP, while mutagenic activity, scored in two *Escherichia coli* strains, seemed much less remarkable.

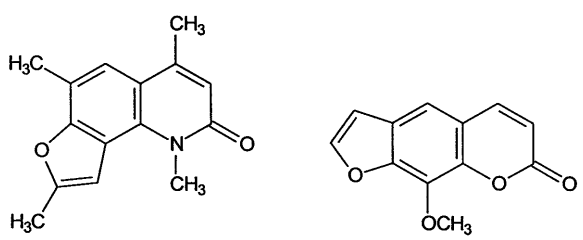
Keywords: Furoquinolinones; Antiproliferative activity; DNA damage

1. Introduction

Furocoumarins, also called psoralens, are photoactive drugs used in PUVA (psoralen plus UVA) therapy to cure several skin diseases [1] and in photopheresis to prevent rejection in organ transplants and to treat T-cell lymphoma and various autoimmune diseases [2].

In spite of the high efficacy of PUVA, this therapy showed some severe side effects, such as skin erythemas [1], genotoxicity [3–5], and carcinogenicity [6], mostly attributed to the lesions induced in DNA by furocoumarin sensitization. Indeed, various kinds of lesions are formed, i.e. covalent mono- and diadducts with pyrimidine bases [7], and covalent DNA–protein cross-links (DPC) [8].

Since many authors considered inter-strand cross-links (ISC) responsible for furocoumarin genotoxicity, several monofunctional derivatives have been prepared and studied [9,10]. Among them, a series of angular furoquinolinones appears to be particularly interesting [11], the most active of the group being a tetramethyl derivative, 1,4,6,8-tetramethylfuro[2,3-*h*]quinolin-2(1*H*)-one (FQ) (Fig. 1), which showed a marked antiproliferative activity, both in the dark and by UVA



FQ 8-MOP

Fig. 1. Molecular structure of the reference compounds.

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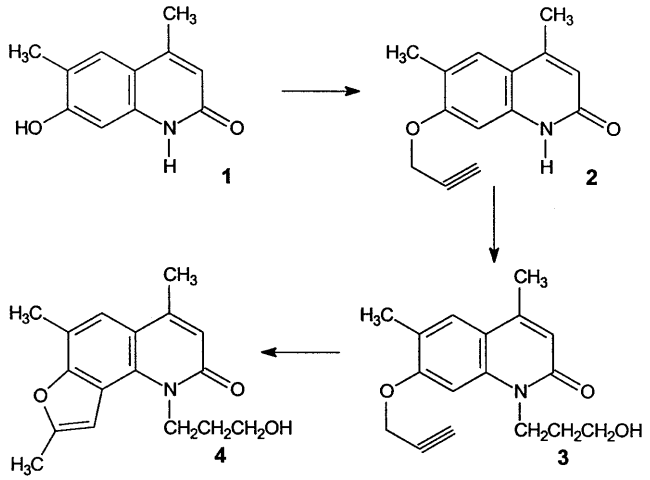

Scheme 1.

activation [12,13]. In the dark, FQ intercalates into DNA and inhibits topoisomerase II. Upon UVA irradiation, FQ induces severe damage into DNA, forming monofunctional adducts but not ISC [11,14]; however, its strong antiproliferative and genotoxic activity in mammalian cells appears to be mostly related to its high capacity of inducing DPC [15].

The FQ behavior appeared to be related to the presence of a methyl group at the nitrogen atom; in fact, when position 1 is free, a pyridone–pyridine tautomerism of the nitrogen nucleus takes place. Therefore, the tetramethylfuroquinolinone, which carries a hydrogen atom at position 1, shows a lower antiproliferative activity, especially in the dark, and a reduced genotoxicity [15]. This tautomerism, which is more favored in the furan-side monoadducts [14], changes the pyridone nucleus into a pyridine one, preventing any further photoreactivity. It has been demonstrated that, similar to ISC [14], DPC is formed by a two-step photoreaction; furan-side monoadducts are formed at first, which react further with proteins to give the DPC [14]. The pyridone–pyridine tautomerism inhibits the second step of DPC induction.

In order to evaluate the influence of bulky substituents in position 1 on biological activity, we planned the synthesis of a new furoquinolinone carrying a substituent larger than a hydrogen atom or a methyl group. In fact, furoquinolinones, similar to furocoumarins, intercalate into DNA forming a molecular complex [11], whose location and status are variously affected by the nature and the hindrance of the substituents. Therefore, we prepared a furoquinolinone derivative substituted at position 1 with a three carbon chain ending with a hydroxyl group, 1-hydroxypropyl-4,6,8 - trimethylfuro[2,3 - *h*]quinolin - 2(1*H*) - one (**4**, HPFQ). We studied some photobiological properties of this novel derivative, in comparison with FQ and 8-MOP (8-methoxypsoralen), as reference drugs (Fig. 1).

2. Chemistry

The synthesis of 1 - (3′ - hydroxypropyl) - 4,6,8 - trimethylfuro[2,3 - *h*]quinolin - 2(1*H*) - one (**4**) was performed according to Scheme 1. The starting material was 4,6-dimethyl-7-hydroxyquinolin-2-one (**1**), previously reported for the synthesis of other heterocyclic systems [16]. Compound **1** was condensed with propargyl chloride to give the corresponding 7-*O*-propargyl ether (**2**), which was reacted with 1-bromo-3-propanol, yielding 4,6-dimethyl-1-(3′-hydroxypropyl)-7-propargyloxyquinolin-2-one (**3**). Compound **3** was finally submitted to Claisen rearrangement in the presence of CsF [17], affording the desired 1-(3′-hydroxypropyl)-4,6,8-trimethylfuro[2,3-*h*]quinolin-2-(1*H*)-one (**4**).

3. Experimental

3.1. Chemistry

Melting points were determined on a Gallenkamp MFB-595-010M melting point apparatus and are uncorrected. Analytical TLC was performed on precoated 60 F_{254} silica gel plates (0.25 mm; Merck) developing with a 9:1 $CHCl_3$–MeOH mixture unless otherwise indicated. Preparative column chromatography was performed using silica gel 60 (0.063–0.100 mm; Merck), eluting with $CHCl_3$. ^1H NMR spectra were recorded on a Varian Gemini-200 spectrometer with TMS as internal standard. The starting complex, 4,6-dimethyl-7-hydroxyquinolin-2-one (**1**), was prepared according to literature methods [16].

3.1.1. 4,6-Dimethyl-7-propargyloxyquinolin-2-one (2)

A mixture of **1** [16] (5.0 g, 26.4 mmol), propargyl chloride (3.4 g, 45.5 mmol) and anhydrous K_2CO_3 (10.0 g) in DMF (150 ml) was heated at 50°C until the starting product disappeared (65 h, TLC). The mixture was diluted with water (300 ml) and the solid was collected, washed with water and crystallized from MeOH to give **2** (3.7 g, 62%). M.p.: 256°C. ^1H NMR ($CDCl_3$): δ 10.56 (s, 1H, –NH), 7.42 (q, 1H, $J = 0.8$ Hz, 5-H), 6.74 (s, 1H, 8-H), 6.42 (q, 1H, $J = 1.1$ Hz, 3-H), 4.82 (d, 2H, $J = 2.4$ Hz, 1′-H), 2.57 (t, 1H, $J = 2.4$ Hz, 3′-H), 2.45 (d, 3H, $J = 1.1$ Hz, 4-Me), 2.30 (d, 3H, $J = 0.8$ Hz, 6-Me). *Anal.* ($C_{14}H_{13}NO_2$) C, H, N.

3.1.2. 4,6-Dimethyl-1-(3′-hydroxypropyl)-7-propargyloxyquinolin-2-one (3)

A mixture of **2** (3.7 g, 16.5 mmol), 3-bromo-1-propanol (12.8 g, 92.5 mmol) and anhydrous K_2CO_3 (10.0 g) in acetone (1.5 l) was refluxed until starting product disappeared (10 days, TLC, 1:1 acetone–ben-

zene). After cooling, the solid was filtered off and washed with fresh acetone. The solvent was evaporated under reduced pressure from the combined filtrate and washings. The residue was purified by column chromatography to give **3** (3.0 g, 66%). M.p.: 184°C. ^{1}H NMR ($CDCl_3$): δ 7.49 (q, 1H, $J = 0.8$ Hz, 5-H), 7.02 (s, 1H, 8-H), 6.49 (q, 1H, $J = 1.1$ Hz, 3-H), 4.86 (d, 2H, $J = 2.4$ Hz, 1′-H), 4.47 (t, 2H, $J = 5.8$ Hz, –N*CH*$_2$CH$_2$CH$_2$OH), 4.31 (t, 1H, $J = 7.0$ Hz, –NCH$_2$CH$_2$CH$_2$*OH*), 3.48 (dt, 2H, $J = 7.0$, 5.8 Hz, –NCH$_2$CH$_2$*CH*$_2$OH), 2.60 (t, 1H, $J = 2.4$ Hz, 3′-H), 2.45 (d, 3H, $J = 1.1$ Hz, 4-Me), 2.31 (d, 3H, $J = 0.8$ Hz, 6-Me), 2.03 (q, 2H, $J = 5.8$ Hz, –NCH$_2$*CH*$_2$CH$_2$OH). *Anal.* ($C_{17}H_{19}NO_3$) C, H, N.

3.1.3. 1-(3′-Hydroxypropyl)-4,6,8-trimethylfuro-[2,3-h]quinolin-2(1H)-one (4)

A mixture of **3** (2.5 g, 8.7 mmol) and CsF (0.8 g, 5.2 mmol) in *N*,*N*-diethylaniline (90 ml) was heated at 210°C until the starting product had disappeared (2 h, ^{1}H NMR). After cooling, the mixture was diluted with AcOEt (200 ml), washed with HCl 1 N (5×100 ml) and water (3×100 ml) and the solvent was evaporated under reduced pressure. The residue was purified by column chromatography to give **4** (1.3 g, 55%). M.p.: 166°C. ^{1}H NMR ($CDCl_3$): δ 7.40 (q, 1H, $J = 0.9$ Hz, 5-H), 6.83 (q, 1H, $J = 1.0$ Hz, 9-H), 6.61 (q, 1H, $J = 1.1$ Hz, 3-H), 4.75 (t, 2H, $J = 5.5$ Hz, –N*CH*$_2$CH$_2$CH$_2$OH), 4.50 (broad s, 1H, –NCH$_2$CH$_2$CH$_2$*OH*), 3.54 (t, 2H, $J = 5.5$, 5.8 Hz, –NCH$_2$CH$_2$*CH*$_2$OH), 2.57 (d, 3H, $J =$ 1.1 Hz, 4-Me), 2.55 (d, 3H, $J = 0.9$ Hz, 6-Me), 2.54 (d, 3H, $J = 1.0$ Hz, 8-Me), 2.07 (q, 2H, $J = 5.5$ Hz, -NCH$_2$*CH*$_2$CH$_2$OH). *Anal.* ($C_{17}H_{19}NO_3$) C, H, N.

3.2. Biology

1,4,6,8-Tetramethylfuro[2,3-*h*]quinolin-2(1*H*)-one (FQ) was prepared by chemical synthesis [11]; 8-MOP was obtained from Chinoin (Milan, Italy). 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 kept at -20°C in the dark. Just before the experiment, a calculated amount of compound solution was added in the dark to phosphate-buffered saline (PBS) or to the growth medium containing cells, to a final DMSO concentration of 0.5%. ^{3}H-thymidine (4.77 TBq mM^{-1}), ^{14}C-thymidine (2.2 GBq mM^{-1}) and the PstI enzyme were from Amersham International Inc. (UK). PM2 DNA were purchased from Boehringer Mannheim GmbH (Germany). *Drosophila melanogaster* embryos were from USB (Amersham Italia S.r.l.). Tetrapropylammonium hydroxide (1 M aqueous solution), L-histidine, nitroblue tetrazolium (NBT) and ethidium bromide (EtBr) were obtained from Sigma-Chemie (Deisenhofen, Germany). *p*-Nitrosodimethylaniline (RNO) were from Carlo Erba (Milan, Italy).

3.2.1. UVA irradiation

DNA solutions and bacterial or mammalian cell suspensions containing the test compound were incubated at room temperature (r.t.) for 15 min in the dark and then put into Petri dishes (5 cm in diameter; 3 ml); control experiments showed that no lesions are formed during this short incubation. The samples were then exposed to UVA light. Samples for alkaline elution were kept in ice during the entire experiment, γ-irradiation included, until the lysis step. UVA exposures were performed with Philips HPW 125 lamps, provided with a built-in Philips filter. Emission was in the 320–400 nm range, with a maximum, over 90% of the total, at 365 nm. Irradiation intensities, determined by a radiometer (mod. 97503, Cole–Parmer Instrument Co., Niles, IL), were as follows: 0.5×10^{-5} W m^{-2}, for lamp placed at distance of 20 cm, and 0.9×10^{-6} W m^{-2}, for lamp placed at distance of 45 cm. In each experiment, controls submitted to 10 kJ m^{-2} of UVA light alone showed no significant difference in comparison with untreated controls (data not shown).

3.2.2. Detection of reactive oxygen species (ROS)

Formation of singlet oxygen was measured according to the method of Kraljic and El Mohsni [18]. Aqueous solutions, containing phosphate buffer (0.02 M, pH 7.3), RNO (4×10^{-5} M), L-histidine (1×10^{-3} M) and the test compound at a concentration of 20 μM were irradiated for increasing times in quartz cuvettes. RNO bleaching was determined by reading the optical density at 440 nm.

To detect the formation of superoxide anion, the method described by Pathak and Joshi was used [19]. Aqueous solutions containing carbonate buffer (pH 10), NBT (1.6×10^{-4}) and the test compound at a concentration of 20 μM were irradiated as before, and the optical density at 560 nm was then determined.

3.2.3. Inhibition of topoisomerase II

The inhibition of topoisomerase II activity was studied using a purified enzyme from *D. melanogaster* embryos [20]. PM2 DNA (0.125 μg) was incubated for 15 min at 30°C in the presence of 2 units of topoisomerase II (1 unit is defined as the activity capable of relaxing 0.3 μg of super-coiled DNA) in the reaction buffer containing 10 mM Tris–HCl (pH 7.9), 50 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 15 μg ml^{-1} BSA and 1 mM ATP. Aliquots of the compound solution (2 μl) were added to reach the following final concentrations: 1, 10, 20, 40 and 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% SDS. Bromophenol blue (2 μl) containing 15% glycerol was added to the samples, which were then analyzed by agarose gel (0.7%) containing TAE (40 mM Tris–sodium acetate, pH 8.2; 1 nM EDTA) for 90 min. The gel was stained for 1 h in aqueous EtBr (0.5 μg ml^{-1}) and then photographed by a Polaroid camera placed over a UV TM36 transilluminator (UVP Inc., San Gabriel, CA).

3.2.4. Detection of ISC

Supercoiled circular DNA of PM2 bacteriophage (4 μg) was linearized by incubating at 37°C for 2 h with PstI restriction enzyme (18 U μl^{-1}) in 100 μl solution containing 10 mM NaCl, 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT and 10 μg BSA. The solution was filtered through a Microcon 100 (Amicon, Beverly, MA), centrifuging at $500 \times g$ for 5 min. The membrane was washed by adding 100 μl of TE (40 mM Tris–HCl, pH 7.5; 1 mM EDTA), and the solution was stirred and centrifuged at $500 \times g$ for a further 5 min. The solution over the membrane was then recovered by inverting the filter and centrifuging at $1000 \times g$ for 3 min. The remaining solution was diluted by adding TE to obtain a DNA concentration of 80 ng μl^{-1} and then stored at 4°C until use. To detect the formation of ISC, PM2-linearized DNA (2 μl of for each sample) was added to the test compound (2 μl; 4× in DMSO) at the appropriate concentration, so that the final molar drug–base pairs ratio was 2. TE (4 μl) was then added to each sample (final volume 8 μl). Two samples of the compound were incubated at r.t. in the dark for 15 min and then exposed to UVA light. As a control, PM2-linearized DNA (2 μl; 80 ng μl^{-1}) was added to DMSO (2 μl) and TE (4 μl) and incubated as described above, but not irradiated. Then 2 μl aliquots of a solution containing 1.5 M sodium acetate and 100 mM EDTA were added to the samples, which were then stirred and centrifuged at $10\,000 \times g$ for 1 min. DNA was precipitated with three volumes (30 μl) of 95% ethanol, and the samples were kept at −80°C for at least 1 h; DNA was recovered by centrifugation at $2000 \times g$ for 20 min at 4°C. Precipitated DNA was washed with ethanol and then suspended TAE (7 μl) containing 0.04% bromophenol blue, 10% glycerol and 30% DMSO, denatured by heating at 90°C for 2 min, and quickly cooled in ice. Samples were analyzed by agarose gel (0.7%) electrophoresis in TAE containing EtBr 0.5 μg ml^{-1} at 50 V. Gels were photographed by a Polaroid camera placed over a UV transilluminator TM36 (UVP Inc., San Gabriel, CA).

3.2.5. DNA synthesis in Ehrlich cells

DNA synthesis was assayed in Ehrlich cells (Lettrè strain) as already described [21]. After UVA irradiation (cell density: 2×10^7 cells ml^{-1} in PBS), the samples (10^6 cells in 0.5 ml of PBS) were incubated for 30 min at 37°C in the presence of 40 kBq ml^{-1} ^{3}H-thymidine. The acid-insoluble fraction was precipitated by 5% ice-cold trichloroacetic acid and collected on Whatman GF/C filters (2.5 cm in diameter). After several washings with cold 1% trichloroacetic acid, the filters were dried and counted. In the experiments carried out in the dark, the cooling and irradiation steps were omitted; the cells were treated with the compound at r.t., incubated for 30 min at 37°C in the dark in the presence of the radioactive precursor, and then processed as above. The results were calculated as the percentage of radioactivity incorporated into the DNA of untreated control cells (ca. 3–6 kBq). Filtrations were carried out using a Sample Manifold apparatus (Millipore Corp., Bedford, MA). Every experiment was carried out at least three times.

3.2.6. Experiments with mammalian cells cultivated in vitro

HeLa cells (kindly provided by Professor Franca Majone, Department of Biology, University of Padova, Italy) were grown in nutrient mixture F-12 Ham medium (Sigma Chemical Co., St. Louis, MO) containing 5% fetal calf serum (increased to 10% in the clonal growth). In both cases media were supplemented with antibiotics and cell growth was accomplished at 37°C in a 5% carbon dioxide atmosphere.

3.2.7. Clonogenic test

HeLa cells ($1.5–2 \times 10^5$) were seeded in Petri dishes in growth medium (4 ml). After 24 h the medium was replaced and the cells exposed to UVA light or incubated for 3 h in the dark according to the selected treatment, as described above. After treatment, aliquots of 200 cells were seeded in growth medium and incubated for 7 days. The colonies were then stained and counted, discarding colonies with less than 50 cells. The efficiency of clonal growth, that is, the ratio between the number of colonies formed and the number of cells seeded, was then calculated. Plating efficiency was about 80%.

3.2.8. Detection of DPC

DPC were detected by alkaline elution performed according to Kohn [22]; each experiment was carried out using an internal standard, i.e. untreated cells labelled with ^{3}H-thymidine and submitted only to a well defined dose of γ-rays, 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^{-1}) or ^{14}C-thymidine (3.7 kBq ml^{-1}). The radioactive medium was removed and was replaced by a fresh one containing the test compound for ^{14}C-cells or 0.5% DMSO for ^{3}H-cells. The cells were exposed to UVA, washed and then submitted to alkaline elution.

About $0.5–1.0 \times 10^6$ ^{14}C-cells were mixed with equal amounts of ^{3}H-cells; the mixture was cooled in ice, exposed to 30 Gy of γ-rays and then deposited on polyvinyl chloride filters (pores 5 μm in diameter; Nucleopore Corp., Pleasanton, CA) in a Swinnex-25 filter holder (Millipore) and immediately lysed with 2% SDS, 0.1 M glycine, 0.025 M EDTA, pH 10 (5 ml). The solution was allowed to flow by gravity, followed by 40 ml of the eluting solution (tetrapropylammonium hydroxide, EDTA, SDS 0.1%, pH 9.6). The elution was carried out using a Gilson Minipuls peristaltic pump, at a flow rate of 0.03–0.04 ml min^{-1}. The fractions were collected with a Gilson fraction collector (approximately, 3.5 ml per fraction) and the radioactivity of both isotopes was then determined.

γ-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 2.5 Gy min^{-1}, as determined by Fricke solution.

3.2.9. Mutagenic test

For mutagenesis we used two *Escherichia coli* WP2 strains, TM6 and TM9, both carrying a nonsense mutation in the *trp*E gene, which is reverted by UV light and base pair substitution mutagens [23]. The TM6 strain is proficient in DNA excision repair, but contains the *R46* plasmid, which codifies an error-prone DNA repair. The TM9 strain contains the same plasmid but is defective in excision repair (*uvr*A).

Bacteria were grown overnight in a minimal Davis–Mingioli salts glucose medium, supplemented with tryptophan (20 mg ml^{-1}). Cells were washed and then suspended in PBS (pH 7.0) containing the test compound (2×10^{-5} M) at a density of 10^8 cells ml^{-1}. Bacteria were irradiated with UVA light, as described for UVA irradiations. For the mutagenesis test, 0.1 ml aliquots of the irradiated suspensions were added to 2 ml of molten 0.6% top agar and poured onto plates containing 20 ml of SEM agar (MMA fortified with 0.1 mg ml^{-1} of Difco nutrient broth). To determine the surviving fraction, the irradiated cells (0.1 ml) were diluted with phosphate buffer, added to 2 ml of molten 0.6% agar, and plated on Davis–Mingioli minimal medium supplemented with tryptophan. The plates were incubated for 48 h at 37°C in the dark and then the colonies were counted. The mutation frequency was expressed as mutants per 10^6 survivors, computed by dividing the number of revertants observed per plate by the number of surviving bacteria at the same treatment and subtracting from the result the number of revertant colonies per 10^6 survivors observed in the controls. All manipulations were performed under red light.

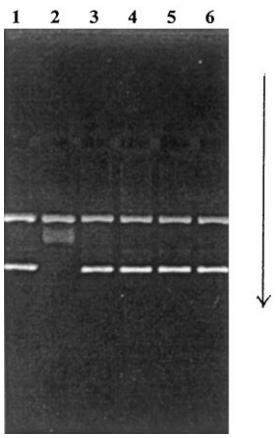

Fig. 2. Inhibition of topoisomerase II activity studied by the relaxation assay. PM2 DNA was incubated in the presence of topoisomerase II and HPFQ or FQ. Agarose gel electrophoresis of the samples is shown. Lane 1: PM2 DNA alone, which migrates as two bands. Lane 2: PM2 DNA incubated in the presence of topoisomerase II, showing only the relaxed form (the slower band). Lanes 3 and 4: PM2 DNA incubated in the presence of topoisomerase II and HPFQ (10 and 1 μM, respectively). Lanes 5 and 6: PM2 DNA incubated in the presence of topoisomerase II and FQ (10 and 1 μM, respectively). The arrow indicates gel mobility.

3.2.10. Skin phototoxicity

Skin phototoxicity was studied on albino guinea pig skin as previously described [24]. Briefly, different amounts of the compounds were applied to the depilated skin as 0.5% methanolic solutions. After 15 min in the dark, the skin was exposed to the same dose of UVA light (5 kJ m^{-2}). The animals were observed for 48 h, checking the minimal drug amount capable of inducing a barely visible erythema.

3.2.11. Radiochemical determination

Filters from macromolecular synthesis determinations were counted using a toluene-based scintillator (PPO 5 g, dimethyl-POPOP 0.25 g, toluene up to 1 l of solution). Fractions from alkaline elution were neutralized with acetic acid and then counted with Ultima Gold XR (Packard Instruments, Meriden, CT) and a Packard Tri-Carb 1900TR spectrometer. Counting was accomplished automatically on the basis of quenching curves obtained using ^{3}H- and ^{14}C-radioactivity standards.

4. Biological results

4.1. In vitro experiments

The HPFQ ability to inhibit topoisomerase II was investigated in vitro using the relaxation test of supercoiled DNA from PM2 phage. The data are reported in Fig. 2 and clearly indicate that HPFQ, like FQ, strongly interferes with the enzyme activity.

With the aim to evaluate HPFQ capacity for damaging cell components, we studied the formation of ISC

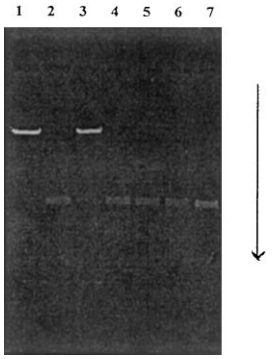

Fig. 3. ISC formation in vitro in linearized PM2 DNA detected by electrophoresis. The drug concentration was 2 molecules per base pair of DNA. 8-MOP was also studied in the same experimental conditions as referred to in the literature. Lane 1: double-stranded DNA. Lane 2: denatured single-stranded DNA. Lane 3: DNA irradiated with 1.5 kJ m^{-2} in the presence of 8-MOP. Lane 4 and 5: DNA treated with HPFQ and exposed to 1.5 and 3 kJ m^{-2}, respectively. Lane 6 and 7: DNA treated with FQ and exposed to 1.5 and 3 kJ m^{-2}, respectively. The arrow indicates the direction of the electrophoretic migration.

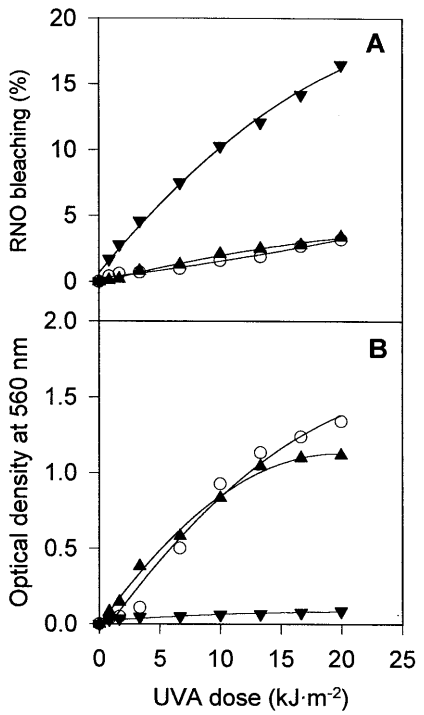

Fig. 4. ROS detection. Panel A: superoxide anion formation by furoquinolinones and 8-MOP. Panel B: singlet oxygen formation by furoquinolinones and 8-MOP. All compounds were used at 20 μM. The symbols are: HPFQ: ○; FQ: ▼; 8-MOP: ▲.

by UVA irradiation, using PM2-linearized DNA. ISC formation was evaluated by gel-electrophoresis separation of single-stranded, not cross-linked DNA, from double-stranded, cross-linked DNA. In this test (Fig. 3) HPFQ, as well as FQ, appeared to be completely incapable of inducing ISC; as expected, 8-MOP formed a considerable amount of ISC.

The HPFQ ability of generating reactive oxygen species (ROS) by UVA irradiation was also evaluated and the results are shown in Fig. 4. HPFQ and 8-MOP produced very low amounts of singlet oxygen, while FQ seemed to be much more effective. Conversely, in the studies on the superoxide anion formation, HPFQ, like 8-MOP, clearly induced detectable levels of this species; as expected, FQ appeared to be practically inactive.

4.2. In vivo experiments

The DNA-damage inducing ability of HPFQ was studied detecting the formation of DPC in HeLa cells by alkaline elution. The results are reported in Fig. 5. DNA from untreated cells eluted quickly, while DNA from sensitized cells generated slower profiles. All compounds gave similar elution profiles, but, while HPFQ and FQ were tested under the same mild experimental conditions (2 μM; 2.5 kJ m^{-2}), 8-MOP was tested under severe ones (20 μM; 10 kJ m^{-2}). So HPFQ and FQ induce DPC to the same extent (about 20×10^{-3} per million of nucleotides), while 8-MOP appears to be about seven times less effective.

The activity of HPFQ on DNA synthesis in Ehrlich cells is shown in Fig. 6. The experiments carried out by simple incubation in the dark are reported in panel A; HPFQ induced a significant inhibition of ^{3}H-thymidine incorporation, which is much lower if compared to FQ. As expected, 8-MOP was ineffective. Conversely, by UVA irradiation (see panel B), HPFQ and FQ generated very similar inhibition curves; in the same experimental conditions, 8-MOP did not induce a significant inhibition of DNA synthesis.

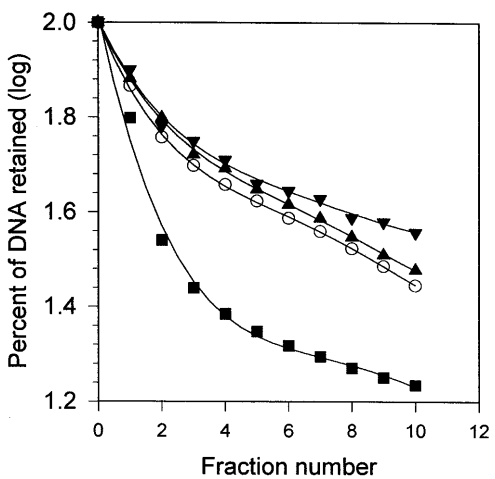

Fig. 5. DPC formation by furoquinolinones and 8-MOP sensitization. HeLa cells were exposed to UVA light in the presence of HPFQ or FQ or 8-MOP and then submitted to alkaline elution. The symbols and the treatments are as follows: untreated controls: ■; HPFQ (2 μM plus 1.5 kJ m^{-2}): ○; FQ (2 μM plus 1.5 kJ m^{-2}): ▼; 8-MOP (5 μM plus 3 kJ m^{-2}): ▲.

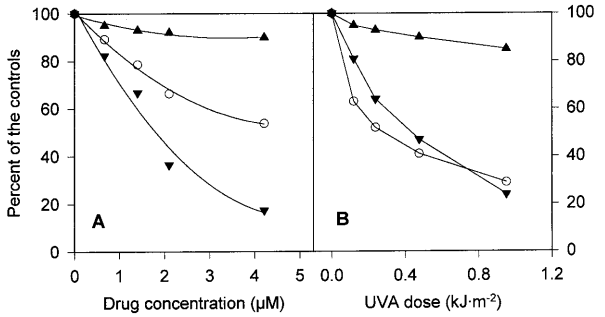

Fig. 6. Inhibition of DNA synthesis in Ehrlich ascite cells. Panel A: by incubation in the dark. Panel B: upon UVA irradiation; in these experiments all compounds were assayed at 2 μM. The symbols are: HPFQ: ○; FQ: ▼; 8-MOP: ▲.

The antiproliferative activity of HPFQ was also evaluated via a clonogenic test in HeLa cells, as reported in Fig. 7. The observed data are similar to those obtained from the DNA synthesis studies, even though FQ, by UVA activation, appeared to be a little more active than HPFQ in the clonogenic test.

To quantify the HPFQ antiproliferative activity, the data from all these experiments were submitted to probit analysis and summarized in Table 1. For the experiments carried out in the dark, the results are expressed as IC_{50} (the drug concentration that induces 50% inhibition of DNA synthesis); for the UVA irradiation, the results are expressed as ID_{50} (the UVA dose that induces 50% inhibition when delivered in the presence of a chosen drug concentration). The two biological substrates showed different sensitivities, i.e. the HPFQ central doses appeared to be different according to the cells used. However, comparing the data obtained with each cell type, we can say that the antiproliferative activity in the dark of HPFQ appears to be one third of FQ. The data obtained by UVA irradiation is very different, as HPFQ showed an antiproliferative activity on both substrates similar to FQ. On the contrary, 8-MOP exhibited 100-fold lower activity.

Table 1
Antiproliferative activity

Comp.	By incubation in the dark $IC_{50} \pm DS$ [a]		By UVA activation $ID_{50} \pm DS$ [b]	
	DNA synthesis	Clonal growth	DNA synthesis	Clonal growth
HPFQ	4.6 ± 0.3	22.5 ± 1.9	0.25 ± 0.02	0.03 ± 0.003
FQ	1.7 ± 0.2	7.2 ± 0.02	0.39 ± 0.02	0.02 ± 0.006
8-MOP	>100	>100	33 ± 1.7	0.38 ± 0.2

[a] IC_{50}: drug concentration (μM) capable of inducing a 50% inhibition of the cell function studied.

[b] ID_{50}: UVA dose (kJ m^{-2}) capable of inducing a 50% inhibition of the cell function studied when delivered in the presence of a 2 μM drug concentration.

Table 2
Threshold doses for erythema induction on guinea pig skin [a]

Comp.	μM cm^{-2}	Relative activity 8-MOP = 1
8-MOP	4.6	1
FQ	3.3	0.72
HPFQ	3.5	0.76

[a] Different amounts of the compounds were applied on the skin as a 0.1% methanolic solution; the skin was then exposed to the same UVA dose, 5 kJ m^{-2}. The animals were then kept in the dark and observed for 3 days.

HPFQ mutagenicity and skin phototoxicity were also evaluated. As shown in Table 2, HPFQ can induce strong erythemas on guinea pig skin, with the same efficiency of FQ and 8-MOP. Fig. 8 shows the results of the mutagenicity test carried out with two *E. coli* strains, TM6 and TM9; the first one is proficient in DNA repair, the second is defective (*uvr*A). These two strains were chosen because they have different sensitivities towards different kinds of DNA lesions: proficient strains are poorly susceptible to monofunctional damage, while bifunctional damage can be easily detected;

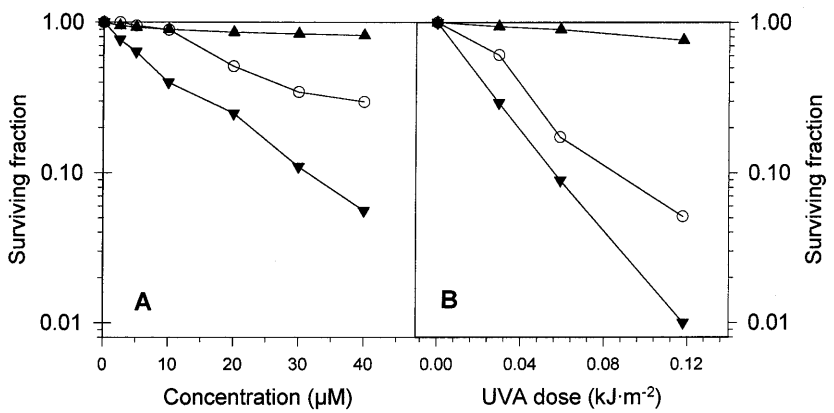

Fig. 7. Clonal growth in HeLa cells. Panel A: by incubation in the dark. Panel B: upon UVA irradiation; in these experiments HPFQ and FQ were assayed at 2 μM, while 8-MOP was used at 5 μM. The symbols are: HPFQ: ○; FQ: ▼; 8-MOP: ▲.

the picture is quite the opposite in deficient strains [25]. In both strains, HPFQ appeared to be less genotoxic than the other two derivatives; in particular, in the defective strain TM9, HPFQ was incapable of forming significant numbers of revertants.

5. Conclusions

The bulkiness of the substituent at nitrogen 1 changed some of the furoquinolinone properties, especially the activity in the dark. In such experimental conditions, HPFQ showed an antiproliferative activity, which appeared to be related to the inhibition of topoisomerase II; however, HPFQ is much less effective in the dark than FQ, with an evident advantage since the interference with such enzyme induces toxic effects [13]; on the other hand this activity is inadequate for potential use as an antitumor drug.

In the experiments carried out by UVA activation in vitro, we only found some small differences between the two furoquinolinones activities; in particular, in the studies on ROS production, HPFQ resembles 8-MOP behavior rather than FQ, showing poor singlet oxygen production and a marked superoxide anion one.

On the contrary, HPFQ resembles FQ in the DNA-damage profile; in fact, both were completely incapable of inducing ISC into DNA, as observed in an in vitro assay, and both induced DPC in mammalian cells to a similar extent. This can explain why HPFQ and FQ have similar biological effects, at least in mammalian systems, as proven by their antiproliferative activities, both in studying DNA synthesis and clonal growth capacity. Moreover, the phototoxic potency of HPFQ and FQ on guinea-pig skin seems to be very similar and comparable to that of 8-MOP. Considering the data obtained studying the damage induced by the two furoquinolinones and 8-MOP, we can observe that the lesions formed by all three compounds are DNA monoadducts and DPCs. As monoadducts were never correlated to the formation of skin erythemas, does this mean that DPCs are implicated in it? At present we cannot give an answer, but we can say that this assumption is consistent with similar results already obtained [12].

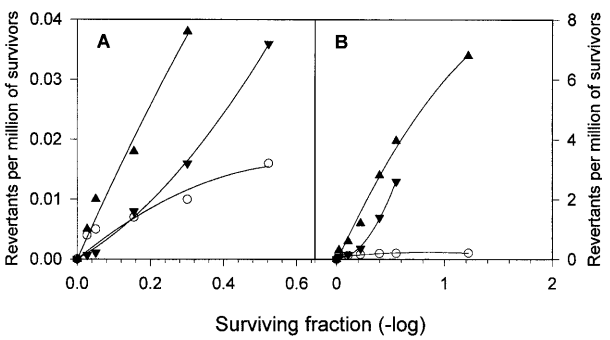

Fig. 8. Mutagenesis in *E. coli* WP2. Panel A: mutagenesis in *E. coli* WP2 TM6, a strain proficient in DNA repair. Panel B: mutagenesis in *E. coli* WP2 TM9, a strain defective in excision repair (*uvr*A$^-$). Bacteria were sensitized by furoquinolinones or 8-MOP (2–4 μM and 0–3 kJ m^{-2}) and survival and revertants were detected. The mutants per million survivors were plotted against the surviving fraction, expressed as the negative value of its logarithm. The symbols are: HPFQ: ○; FQ: ▼; 8-MOP: ▲.

About mutagenic activities in bacteria, HPFQ and FQ gave very different results. We used two *E. coli* WP2 strains, one proficient and another defective in DNA repair, with the aim to emphasize the revertant production. In both strains HPFQ appeared to be poorly active; in the defective strain TM9, revertants induced by HPFQ were barely detectable. Conversely, FQ and 8-MOP appeared to be active in both strains.

These results can hardly be understood in the light of the latest data. It has already been observed that 8-MOP and FQ are incapable of inducing DPC in bacteria [15]; therefore it is reasonable to suppose that HPFQ is also incapable. On the other hand, 8-MOP, unlike HPFQ and FQ, induces ISC, while in ROS formation it behaves like HPFQ rather than FQ. Consequently, the differences observed in the revertant formation should be related to monoadducts formed by HPFQ. To prove these results, this point should be investigated by isolating HPFQ monoadducts and verifying their structures.

In conclusion, HPFQ represents a new photosensitizing furoquinolinone showing a marked antiproliferative activity, with a reduced cytotoxicity in the dark and poor genotoxicity under UVA irradiation.

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