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DNA Damage and Biological Effects Induced by Photosensitization with New N_1-Unsubstituted Furo[2,3-*h*]quinolin-2(1*H*)-ones

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Abstract—New furoquinolinones unsubstituted at the N_1 position were prepared and their photobiological activities were studied in comparison with 4,6,8,9-tetramethylfuro[2,3-*h*]quinolin-2(1*H*)-one (HFQ) and 8-MOP. The anti-proliferative activity of furoquinolinones **3a**–**f** was tested upon UVA irradiation in mammalian cells, studying DNA synthesis and clonal growth capacity, and in micro-organisms, evaluating T2 infectivity. Almost all compounds appeared to be more active than 8-MOP, and free of any mutagenic activity and skin phototoxicity. Among them, compound **3b** was the most effective one. Similarly to HFQ, compound **3b** appeared to be very active also in DNA damaging, forming monoadducts and $DPC_{L=0}$, but no ISC and $DPC_{L>0}$, both responsible for furocoumarin genotoxicity and phototoxicity. Moreover, Ehrlich ascites cells, photoinactivated by the new furoquinolinone **3b** and injected into recipient mice, proved to be capable of inducing protection against a successive challenge performed with the same tumor cells. For all these features, **3b** seemed to be a new promising potential drug for PUVA therapy and photopheresis.

Introduction

Linear furocoumarins (psoralens) are active sensitizers widely used in PUVA (Psoralen plus UVA) therapy for treatment of several skin diseases,[1] and in photopheresis, for preventing rejection in organ transplantation, to treat T-cell lymphoma and other autoimmune diseases.[2] The compounds currently used are 8-methoxypsoralen (8-MOP), 5-methoxypsoralen (5-MOP) or 4,5′,8-trimethylpsoralen (TMP) (Fig. 1). Nevertheless, their therapeutic use shows various toxic effects, such as severe skin erythemas,[1] and genotoxicity, with induction of point mutations[3,4] and chromosomal aberrations.[5] In addition, PUVA therapy is associated to a certain risk of skin cancer.[6] Various authors attributed these dangerous effects to the severe damage induced into DNA by furocoumarin sensitization.[7] In fact psoralens form covalent monoadducts (MA) with pyrimidine bases, or diadducts: covalent inter-strands cross-links (ISC) with two pyrimidine bases on the opposite DNA strands,[7] and covalent DNA-protein cross-links (DPC).[8]

With the aim to obtain more effective and less toxic drugs for PUVA therapy and photopheresis, we have prepared and studied various angular compounds, unable to induce ISC for geometrical reasons, such as angelicin derivatives,[9] and recently some angelicin bioisosters, namely furoquinolinones.[10–12] Among them, 1,4,6,8-tetramethylfuro[2,3-*h*]quinolin-2(1*H*)-one (FQ), and 4,6,8,9-tetramethylfuro[2,3-*h*]quinolin-2(1*H*)-one (HFQ) are the more interesting (Fig. 1). Both compounds are characterized by a very strong photosensitizing activity. However, FQ also shows an evident skin phototoxicity and a marked clastogenic activity,[11] while these side effects are reduced or absent with HFQ.[13,14] Both derivatives induce large amounts of MA and DPC, without forming ISC. Moreover, the formation and the structures of DPC seemed to be different for the two compounds. In fact, DPC_{FQ} are real bifunctional adducts, in which the FQ molecule is the covalent linkage between DNA and proteins, and so, they are defined *DPC at length greater than zero* ($DPC_{L>0}$).[13] Similarly to ISC, these DPC are formed by a two-step reaction, first forming a furan-side MA between FQ and DNA pyrimidine base, and then reacting with proteins to yield DPC. Applying the well-known

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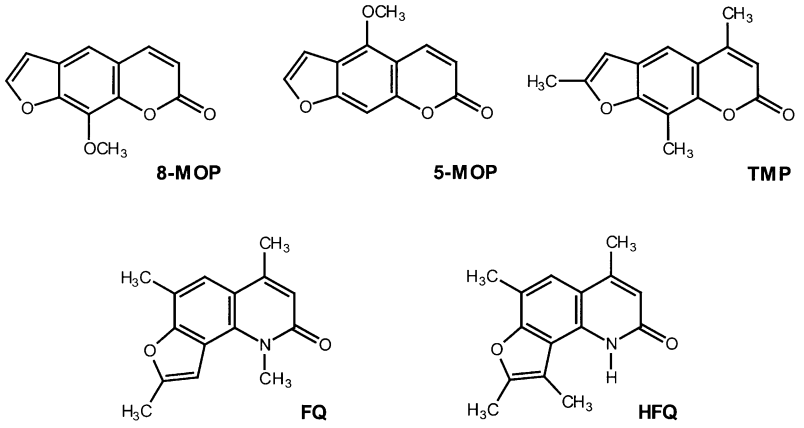
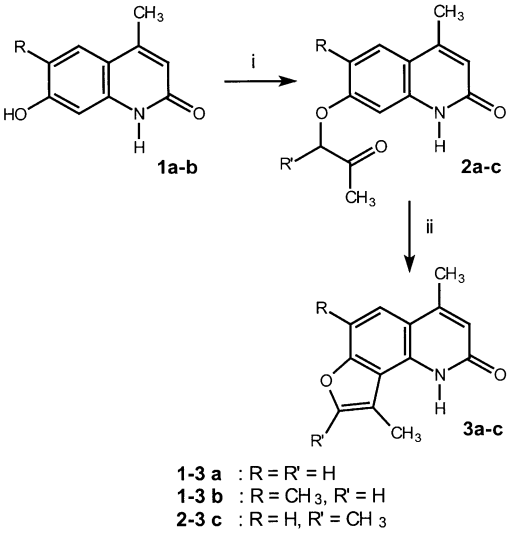


Figure 1. Structure of 8-methoxypsoralen (8-MOP), 5-methoxypsoralen (5-MOP), 4,5′,8-trimethylpsoralen (TMP), 1,4,6,8-tetramethylfuro[2,3-*h*]quinolin-2(1*H*)-one (FQ), and 4,6,8,9-tetramethylfuro[2,3-*h*]quinolin-2(1*H*)-one (HFQ).

double irradiation technique to FQ, we observed that $DPC_{L>0}$ are responsible for its very high clastogenic activity.[11]

In contrast, HFQ induces DPC without participating in the reaction and MA_{HFQ} proved to be unconvertible into diadducts.[13] These lesions, in which DNA and proteins are directly linked together, resemble DPC induced by UVC (*DPC at zero length,* $DPC_{L=0}$). Since HFQ induces MA and $DPC_{L=0}$ to a comparable extent with FQ, the different nature of DPC is probably responsible for the different toxicities of these two furoquinolinones.

Since the absence of N_1-substituent seems to make MA_{HFQ} unable to convert into diadducts, due to the tautomerism pyridone–pyridine,[14] we synthesized a series of furoquinolinones unsubstituted at N_1 position. We also studied some aspects of their potential therapeutic and toxic activity on various biological substrates, using HFQ and 8-MOP as reference compounds.

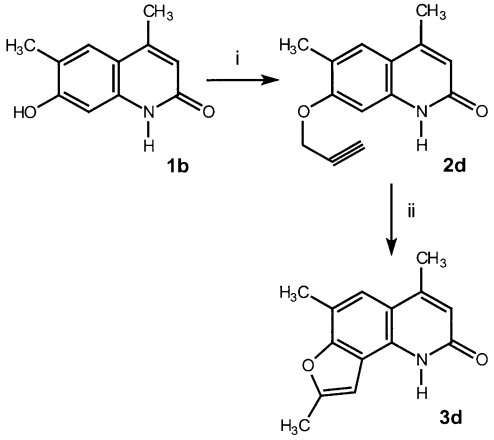


Scheme 1. Reagents and conditions: (i) $CH_3COCH(R')Cl$, acetone, K_2CO_3, reflux, 45–68%; (ii) toluene, methanesulfonic acid, reflux, 42–53%.

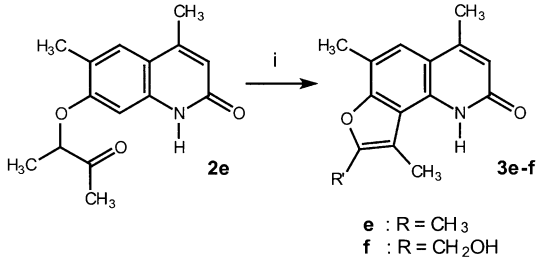
Results and Discussion

Chemistry

The title compounds were synthesized according to the pathways summarized in Schemes 1–3, which differed for the cyclization conditions used to build the furan ring. Starting products were methyl-7-hydroxyquinolin-2-ones **1a**–**b**,[15] which were condensed with chloroacetone or chlorobutanone or propargyl chloride to give the corresponding 7-*O*-oxoalkyl or 7-*O*-alkynyl ethers **2a**–**d**. Compounds **2a**–**c** were submitted to cyclization in



Scheme 2. Reagents and conditions: (i) $CH{\equiv}CCH_2Cl$, acetone, K_2CO_3, reflux, 40%; (ii) *N*,*N*-diethylaniline, CsF, 210 °C, 10%.



Scheme 3. Reagents and conditions: (i) concd H_2SO_4, 50 °C, 32% (**5a**) and 36% (**5b**).

Table 1. Biological activity of the new furoquinolinones

Compound	Antiproliferative activity			Mutations in *E. coli TM9*[d]	Skin phototoxicity[e]
	DNA synthesis in Ehrlich cells[a]	Clonal growth of HeLa cells[b]	T2 phage inactivation[c]		
8-MOP	3.7±0.20	0.790±0.020	0.80±0.110	2.96±0.12	+ + +
HFQ (**3e**)	0.40±0.02	0.025±0.004	0.04±0.001	0.04±0.20	±
3a	7.74±1.20	0.350±0.020	0.25±0.040	0.23±0.03	−
3b	0.80±0.60	0.018±0.005	0.08±0.002	0.23±0.01	−
3c	1.43±0.20	0.140±0.010	0.40±0.060	0.16±0.01	−
3d	2.71±0.18	1.450±0.070	1.65±0.200	0.44±0.05	−
3f	3.43±0.51	0.058±0.020	0.47±0.070	0.31±0.03	−

[a] ID_{50}±SD: UVA dose which reduces to 50% DNA synthesis in Ehrlich cells when delivered in the presence of 20 μM drug concentration.
[b] ID_{50}±SD: UVA dose which reduces to 50% clonal growth of HeLa cells when delivered in the presence of 5 μM drug concentration.
[c] ID_{37}±SD: UVA dose which reduces to 37% the surviving fraction when delivered in the presence of 2 μM drug concentration.
[d] Revertants per 10^6 survivors scored at 0.37 surviving fraction.
[e] Phototoxicity on guinea-pig skin at 0.5 mM cm^{-1} drug concentration and 20 kJ m^{-2} UVA dose. Symbols: −no erythema, ±light erythema, + + + strong erythema.

toluene in the presence of methanesulfonic acid, yielding the corresponding methylfuro[2,3-*h*]quinolin-2(1*H*)-ones **3a**–**c**, while compound **2d** was submitted to Claisen rearrangement in the presence of CsF,[16] affording 4,6,8-trimethylfuro[2,3-*h*]quinolin-2(1*H*)-one (**3d**).

Since methylfuroquinolin-2-one derivatives were already been synthetized using concentrated sulfuric acid to cyclize the furan ring,[10] we tried to use the same experimental conditions for the ethers **2a**–**c**. In concentrated sulfuric acid only compound **2c** gave the desired furoquinolin-2-one **3c**, but with lower yield as compared with the method above mentioned, while compounds **2a** and **b** gave no cyclization products. Compound **2e** in concentrated sulfuric acid at room temperature gave only 4,6,8,9-tetramethylfuro[2,3-*h*]quinolin-2(1*H*)-one (**3e**),[10] while at 50 °C gave also a comparable amount of 8-hydroxymethyl-4,6,9-trimethylfuro[2,3-*h*]quinolin-2(1*H*)-one (**3f**), deriving from the oxidation of the methyl group in the 8 position. The same result was also obtained directly treating compound **3e** with concentrated sulfuric acid at 50 °C, obtaining derivative **3f**, together with many co-products, especially if the reaction time was prolonged until the starting product disappeared. Since there were two methyl groups in the furan ring, ^{13}C NMR measurements were performed to demonstrate that the oxidation occurred only at the 8 position. ^{13}C NMR spectra, obtained by retaining the proton–carbon couplings through gated decoupling experiments, clearly showed that the C-9 molteplicity was the same for compounds **3e** and **3f**, while for compound **3e** the C-8 was coupled with three protons (quartet at 150.03 ppm), and for compound **3f** the C-8 was coupled with two protons (triplet at 149.73 ppm), so the hydroxymethyl group was in the 8 position.

Biology

Experiments for a preliminary screening. The antiproliferative activity of furoquinolinones **3a**–**f** was tested upon UVA irradiation in mammalian cells, studying the effect on DNA synthesis in Ehrlich cells, that is a short term effect, and the clonal growth capacity of HeLa cells, that is a long-term effect. As summarized in Table 1, all derivatives showed a significant activity, higher than 8-MOP in most cases; compound **3b** appeared to be the best one, with data comparable to those of HFQ.

The anti-proliferative activity was also tested in microorganisms, by examining T2 phage inactivation, a very useful system to detect DNA damage.[17] As shown in Table 1, again compound **3b** was the most effective.

The mutagenic ability was assayed in *Escherichia coli TM9*, a bacterial strain defective in DNA repair and very sensitive to mutagens (Table 1). All new furoquinolinones seemed one order of magnitude less effective than 8-MOP, classified as a mutagen for the high numbers of revertants induced.

Finally, skin phototoxic properties was determined on guinea pig (Table 1); all derivatives appeared to be completely unable to form erythemas.

Experiments carried out with compound 3b. Since the most active and interesting derivative seemed to be compound **3b**, its photobiological properties were carefully investigated. Also in these experiments HFQ and 8-MOP were chosen as references.

The ability to induce DNA damage was tested in vitro by exposing **3b** to increasing UVA light doses and recording the corresponding UV spectra (Fig. 2). As UVA dose increased, a marked decrease of optical density at 250 nm was observed, but no modifications at 335 nm, and only moderate increases at 270–320 and 350–400 nm, without wavelength shifts of absorption picks. These results suggested that **3b** probably broke down forming many degradation products, but not one defined compound.

As compound **3b** showed high emission intensities in the characteristic coumarin region (300–380 nm) of the UV spectrum, its ability to photoreact with DNA was assayed by fluorescence determinations (Table 2). No significant modifications were detected in fluorescence spectrum after exposure of **3b** aqueous solution to UVA

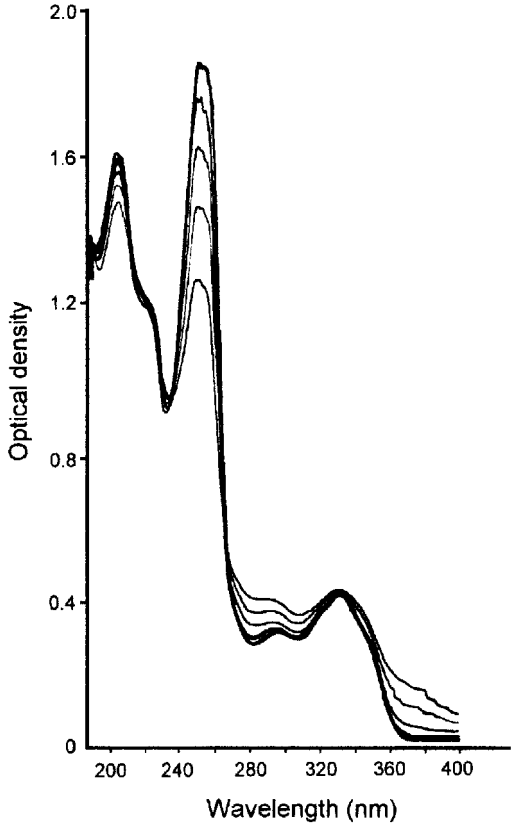


Figure 2. UV spectrum of compound **3b** exposed to increasing UVA light doses: 0 (thick line), 0.8, 1.6, 3.2, 6.4, 12.8 and 25.6 kJ m^{-2}.

Table 2. Formation of furan side MA with calf thymus DNA in vitro

	UVA dose (kJ m^{-2})	λ_{max} emission (nm)	% Fluorescence
3b alone[a]	0	400	60±2.1
	10	400	51±2.3
DNA alone[a]	10	260	49±2.1
3b plus DNA[b]	0	350–450	4±1.5
	2.5	366	33±0.8
	5	366	43±1.1
	10	366	48±1.1

[a]Solutions were exposed to UVA light and then the fluorescence was determined.
[b]Solutions were exposed to UVA light, DNA was precipitated and dissolved in the initial volume of water and the fluorescence was then determined.

Table 3. DNA damage in HeLa cells

Compound	ISC[a]	DPC[a]	DSB[b]
8-MOP	4.5±0.51	2.05±0.12	14.1±0.80
HFQ	0.01±0.001	5.25±1.20	3.1±0.10
3b	0.01±0.001	7.46±0.91	5.5±0.12

[a]Number of the lesions ($\times 10^{-3}$) per 10^6 nucleotides, ±standard deviation, detected by alkaline elution after UVA irradiation (1 kJ·m^{-2}) in the presence of 1 μM drug.
[b]Percent of DNA fragmentation ± standard deviation, detected by neutral elution after UVA irradiation (0.67 kJ m^{-2}) in the presence of 2 μM drug followed by 24 h incubation in growth medium.

light, as well as for aqueous solutions of calf thymus DNA, which gave always a fluorescence peak at 260 nm. After exposure of aqueous solution of DNA containing **3b** to increasing UVA doses, a second fluorescence peak at 366 nm appeared in the spectrum, with intensity increasing with UVA doses. This was attributed to the formation of fluorescent furan-side monoadducts into DNA.

The formation of ISC and DPC was studied in HeLa cells by alkaline elution.[18] As shown in Table 3, furoquinolinone **3b** appeared to be completely unable to induce ISC, like HFQ, due to the angular molecular structure, which prevents the formation of such lesions.[14] This finding was consistent with the low mutagenic activity shown in *E. coli TM9*. On the contrary, 8-MOP formed high levels of ISC, as expected. Moreover, compound **3b** induced noticeable amounts of DPC, larger than 8-MOP and HFQ. This evidence can explain the strong antiproliferative activity of **3b**.

The formation of double-strand breaks (DSB) was also studied in HeLa cells using neutral elution[18] (Table 3). When cells were submitted to DSB detection just after sensitization, always negative results were scored. DNA fragmentation was observed only when cells were incubated for a suitable time in growth medium after sensitization. Even in these experimental conditions, compound **3b** and HFQ induced only moderate DSB amounts, while 8-MOP yielded a very large DNA fragmentation. This result meant that DSB were probably due to an enzymatic repair of the lesions induced into DNA and not only to a simple photochemical event.

All these data suggested that the biological activity of **3b** could be correlated to the formation of MA and DPC.

To investigate the mechanism and the nature of DPC induced by **3b**, some experiments based on the double irradiation protocol were carried out. By means of this technique, MA or diadducts were subsequently induced, as they required the absorption of one or two photons, respectively. Moreover, this method allowed to discriminate between $DPC_{L>0}$ (requiring a biphotonic reaction) and $DPC_{L=0}$ (formed by a monophotonic one).[13] This procedure thus represented a practical way of studying the biological consequences of these different DNA lesions. Compound **3b** could only induce $DPC_{L=0}$ into DNA, since it was unable to form ISC; therefore, evaluating its antiproliferative activity before and after the second irradiation step, we could get information on its mechanism of DPC induction. The results obtained studying the clonal growth capacity of HeLa cells, using the double irradiation method, are shown in Figure 3. Panel A reports the data related to compound **3b** and HFQ; when the unbound drug was not removed after the first irradiation step, the inhibition curves decreased according to the increase of UVA doses delivered during the second step. On the contrary, when cells were washed after the first irradiation step, the second one did not reduce significantly the surviving fraction. These results suggested that both compounds formed MA incapable of further reacting and

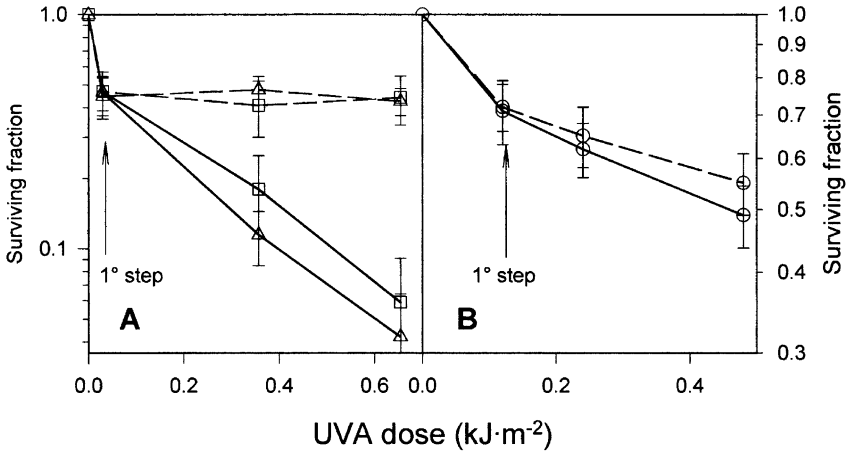


Figure 3. Clonal growth of HeLa cells after sensitization with the double irradiation method. Cells were exposed to UVA light in the presence of **3b** (2 μM), HFQ (2 μM), and 8-MOP (5 μM). The irradiated samples were washed with PBS (dotted lines) or not washed (solid lines), and then submitted to the second irradiation step performed with increasing UVA doses. Clonal growth capacity was determined. Symbols: **3b**, Δ; HFQ, □; 8-MOP, ○. Panel A: first irradiation step: 0.03 kJ m^{-2}; Panel B: first irradiation step: 0.12 kJ m^{-2}.

Table 4. Inhibition of the tumor transfer capacity of Ehrlich cells by UVA irradiation in the presence of compound **3b**

Drug concentration (μM)	UVA dose (KJ m^{-2})	% Mortality	Mean survival time ± SD (days)
0	0	100	10 ± 3.0
0	2.66	100	10 ± 2.4
15	0	100	10 ± 1.6
15	2.66	0	>90
2.5	2.66	0	>90

Ehrlich cells were sensitized and then injected ip into health NCL mice (5×10^6 cells per animal). The animals (groups of 10) were observed for 90 days.

Table 5. Protection against the transplant of viable Ehrlich cells

Protecting treatment (cells×10^6)[a]	Challenge		% Mortality[b]	Mean survival time (days)
	Days	Viable cells (×10^6)		
—	—	0.3	100	10.1 ± 2.1
—	—	1.0	100	9.7 ± 3
5	10	0.3	100	11 ± 1.3
5	21	0.3	0	>90
5	21	1.0	0	>90

[a]Protection was always performed by injecting 5×10^6 cells previously submitted to **3b** sensitization (2.5 μM; 2.66 kJ m^{-2}).
[b]The recipient animals (groups of 20) were observed for 90 days.

consequently forming only $DPC_{L=0}$. Panel B showed the data obtained with 8-MOP, which formed ISC and $DPC_{L>0}$ both responsible for antiproliferative effects;[13,14] in this case, after the first irradiation step, the inhibition curves generated removing and not removing the unbound sensitizer are quite similar.

Finally, experiments were carried out with mice in vivo to investigate the tumor transmission behavior of **3b**. Ehrlich cells were submitted to **3b** photosensitization and then injected intraperitoneously into healthy mice, which were then observed scoring the tumor growth (Table 4). Untreated cells could transmit the tumor very efficiently, since all animals developed the disease and died after few days. In contrast, sensitized cells completely lost this property: in fact, after 90 days all the treated animals survived. This suggested that the sensitized cells became completely unable to replicate.

Then, it was investigated if the injection of so inactivated cells could induce in mice a protective effect against a successive challenge performed by viable Ehrlich cells (Table 5). When untreated mice received $0.3–1\times10^6$ viable Ehrlich cells, they developed the tumor and died within a short time; the same result was obtained with mice injected with 5×10^6 photoinactivated cells 10 days before the challenge with $0.3–1\times10^6$ viable cells. However, when this challenge was performed after 21 days from the injection of the inactivated cells, all animals survived, so showing an acquired resistance against tumor development.

These data could be explained supposing that **3b** sensitization could modify the antigenic character of the photoinactivated cells, so that the injected mice developed an immunological reaction against the same viable tumor cells.

It was interesting to observe that compound **3b** could be considered as an isoster of 4,6,4′-trimetilangelicin (TMA), a well-known angelicin derivative, presumably the most effective one; the only difference was that the δ-lactonic ring of TMA was replaced by a δ-lactamic one. The comparison between the photobiological properties of these two derivatives was very stimulating. Both compounds efficiently photoreacted with DNA: however **3b** did not form ISC at all, while TMA could induce moderate but significant amounts of ISC.[19] Compound **3b** produced numbers of $DPC_{L=0}$, while TMA induced low levels of DPC of no established type.[8,20] Therefore, these two compounds induced different bifunctional lesions. We must remember that TMA proved to be genotoxic in various bacterial strains

and in mammalian cells.[21] On the other side, from the preliminary data until now available, **3b** genotoxicity seemed to be very reduced in comparison with TMA and with 8-MOP too. So this was more evidence that different kinds of DNA damage induce different biological consequences.

Conclusion

A new series of furoquinolinones lacking the substituent at the N_1 position was prepared and studied, in order to confirm the interesting properties of the parent HFQ, and with the aim to obtain new agents for PUVA therapy and photopheresis with improved features, that is low levels of clastogenicity and skin phototoxicity.

Among the furoquinolinones synthesized, compound **3b** appeared very promising. It demonstrated highly effective in inducing DNA damage, without forming ISC and $DPC_{L>0}$, both responsible for the genotoxicity and phototoxicity of furocoumarins, and so showing very strong antiproliferative activity. In addition, compound **3b** showed no mutagenic effects and absence of skin erythema.

Moreover, this new furoquinolinone proved to be able to protect against tumor growth, so predicting its potential use in photopheresis: in this therapy lymphocytes taken from the patients and specific for the reaction of organ rejection after transplant, are modified by furocoumarin sensitization, and therefore are recognized by the patient immune system as a not-self, thus performing a vaccination against the same rejection process.

All these findings require further studies to improve the knowledge of the photobiological properties of compounds **3b**, which seems to be a very promising agent for PUVA therapy and photopheresis.

Experimental

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 $CHCl_3$/MeOH mixture (9:1) unless otherwise indicated. Preparative column chromatography was performed using silica gel 60 (0.063–0.100 mm; Merck), eluting with a $CHCl_3$/MeOH mixture (98:2). ^1H NMR spectra were recorded on a Varian Gemini-200 spectrometer and ^{13}C NMR were recorded on a Bruker AMX300 spectrometer, with TMS as internal standard. Mass spectra were recorded on a Varian MAT112 spectrometer. Elemental analyses were obtained on all intermediates and were within $\pm 0.4\%$ of theoretical values. Starting 4-methyl-7-hydroxyquinolin-2-one **1a** and 4,6-dimethyl-7-hydroxyquinolin-2-one **1b** were prepared according to literature methods,[15] as well as 4,6-dimethyl-7-(2′-oxopropyloxy)quinolin-2-one **2e**.[10]

7-*O*-Ethers. General procedure

7-(2′-Oxopropyloxy)quinolin-2-ones and 7-[2′-(3′-oxo)-butyloxy]quinolin-2-ones (2a–c). General procedure. A mixture of **1** (20.0 mmol), chloroacetone or 3-chloro-2-butanone or propargyl chloride (24.0 mmol) and anhydrous K_2CO_3 (10.0 g) in acetone (500 mL) was refluxed until **1** disappeared (40 h, TLC). After cooling, the solid was filtered off and washed with fresh acetone. The solvent was evaporated from the combined filtrate and washings and the residue was crystallized from MeOH to give **2**.

4-Methyl-7-(2′-oxopropyloxy)quinolin-2-one (2a). Yield 54%; mp 206 °C; ^1H NMR ($CDCl_3$) δ 7.61 (d, 1H, $J = 8.9$ Hz, 5-H), 6.87 (dd, 1H, $J = 8.9$, 2.5 Hz, 6-H), 6.80 (d, 1H, $J = 2.5$ Hz, 8-H), 6.46 (q, 1H, $J = 1.2$ Hz, 3-H), 4.68 (s, 2H, 1′-H), 2.48 (d, 3H, $J = 1.2$ Hz, 4-Me), 2.34 (s, 3H, 3′-H); anal. ($C_{13}H_{13}NO_3$): C, H, N.

4,6-Dimethyl-7-(2′-oxopropyloxy)quinolin-2-one (2b). Yield 68%; mp 248 °C; ^1H NMR ($CDCl_3$) δ 7.49 (s, 1H, 5-H), 6.61 (s, 1H, 8-H), 6.21 (q, 1H, $J = 1.1$ Hz, 3-H), 4.83 (s, 2H, 1′-H), 2.37 (d, 3H, $J = 1.1$ Hz, 4-Me), 2.26 (s, 3H, 6-Me), 2.23 (s, 3H, 3′-H); anal. ($C_{14}H_{15}NO_3$): C, H, N.

4-Methyl-7-[2′-(3′-oxo)butyloxy]quinolin-2-one (2c). Yield 45%; mp 186 °C; ^1H NMR ($CDCl_3$) δ 7.59 (d, 1H, $J = 8.9$ Hz, 5-H), 6.79 (dd, 1H, $J = 8.9$, 2.5 Hz, 6-H), 6.76 (d, 1H, $J = 2.5$ Hz, 8-H), 6.44 (q, 1H, $J = 1.1$ Hz, 3-H), 4.83 (q, 1H, $J = 6.9$ Hz, 2′-H), 2.46 (d, 3H, $J = 1.1$ Hz, 4-Me), 2.22 (s, 3H, 4′-H). 1.55 (d, 3H, $J = 6.9$ Hz, 1′-H); anal. ($C_{14}H_{15}NO_3$): C, H, N.

4,6-Dimethyl-7-propargyloxyquinolin-2-one (2d). Yield 40%; mp 255 °C; ^1H NMR ($CDCl_3$) δ 7.42 (s, 1H, 5-H), 6.72 (s, 1H, 8-H), 6.41 (q, 1H, $J = 1.0$ Hz, 3-H), 4.81 (d, 2H, $J = 2.4$ Hz, 1′-H), 2.57 (t, 3H, $J = 2.4$ Hz, 3′-H), 2.45 (d, 3H, $J = 1.0$ Hz, 4-Me), 2.30 (s, 3H, 6-Me); anal. ($C_{14}H_{13}NO_2$): C, H, N.

Methylfuro[2,3-*h*]quinolin-2(1*H*)-ones (3a–c). General procedure. A mixture of **2** (10.0 mmol) and methanesulfonic acid (40.0 mmol) in toluene (500 mL) was refluxed until **2** disappeared (40–60 h, TLC). After cooling, the reaction mixture was washed with water (2×200 mL) and the organic phase was evaporated under reduced pressure. The residue was crystallized from MeOH to give **3**.

4,9-Dimethylfuro[2,3-*h*]quinolin-2(1*H*)-one (3a). Yield 53%; mp 292 °C; ^1H NMR ($CDCl_3$) δ 7.57 (d, 1H, $J = 8.9$ Hz, 5-H), 7.46 (q, 1H, $J = 1.1$ Hz, 8-H), 7.34 (d, 1H, $J = 8.9$ Hz, 6-H), 6.50 (q, 1H, $J = 1.2$ Hz, 3-H), 2.58 (d, 3H, $J = 1.2$ Hz, 4-Me), 2.44 (d, 3H, $J = 1.1$ Hz, 9-Me); MS (EI) m/z 213 (M^+); anal. ($C_{13}H_{11}NO_2$): C, H, N.

4,6,9-Trimethylfuro[2,3-*h*]quinolin-2(1*H*)-one (3b). Yield 42%; mp 224 °C; ^1H NMR ($CDCl_3$) δ 7.46 (q, 1H, $J = 1.1$ Hz, 8-H), 7.35 (q, 1H, $J = 0.9$ Hz, 5-H), 6.49 (q, 1H, $J = 1.2$ Hz, 3-H), 2.57 (d, 3H, $J = 1.2$ Hz, 4-Me), 2.54 (d, 3H, $J = 0.9$ Hz, 6-Me), 2.53 (d, 3H, $J = 1.1$ Hz, 9-Me); MS (EI) m/z 227 (M^+); anal. ($C_{14}H_{13}NO_2$): C, H, N.

4,8,9-Trimethylfuro[2,3-*h*]quinolin-2(1*H*)-one (3c). Yield 47%; mp 233 °C; ^{1}H NMR ($CDCl_3$) δ 7.41 (d, 1H, J = 8.9 Hz, 5-H), 7.16 (d, 1H, J = 8.9 Hz, 6-H), 6.32 (q, 1H, J = 1.1 Hz, 3-H), 2.44 (d, 3H, J = 1.1 Hz, 4-Me), 2.35 (q, 3H, J = 0.8 Hz, 8-Me or 9-Me), 2.33 (q, 3H, J = 0.8 Hz, 8-Me or 9-Me); MS (EI) m/z 227 (M^+); anal. ($C_{14}H_{13}NO_2$): C, H, N.

4,6,8-Trimethylfuro[2,3-*h*]quinolin-2(1*H*)-one (3d). A mixture of **2d** (6.4 g, 28.0 mmol) and CsF (3.2 g, 20.4 mmol) in *N,N*-diethylaniline (150 mL) was heated at 210 °C until starting product 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 **3d** (0.64 g, 10%): mp > 300 °C; ^{1}H NMR ($CDCl_3$) δ 7.29 (q, 1H, J = 0.8 Hz, 5-H), 7.02 (q, 1H, J = 1.0 Hz, 9-H), 6.55 (q, 1H, J = 1.0 Hz, 3-H), 2.56 (d, 6H, J = 1.0 Hz, 4-Me and 8-Me), 2.55 (d, 3H, J = 0.9 Hz, 6-Me); MS (EI) m/z 227 (M^+); anal. ($C_{14}H_{13}NO_2$): C, H, N.

4,6,8,9-Tetramethylfuro[2,3-*h*]quinolin-2(1*H*)-one (3e) and 8-hydroxymethyl-4,6,9-trimethylfuro[2,3-*h*]quinolin-2(1*H*)-one (3f). Compound **2e**[10] (2.0 g, 7.7 mmol) was dissolved in concd. H_2SO_4 (50 mL) and the solution was heated at 50 °C for 1 h. The mixture was poured into cold water (500 mL) and the obtained precipitate was collected, washed with water and purified by column chromatography to give **3e** (0.6 g, 32%), followed by **3f** (0.7 g, 36%).

Compound 3e:[10]. Mp 231 °C (MeOH); ^{1}H NMR (CD_3OD) δ 7.19 (q, 1H, J = 0.9 Hz, 5-H), 6.32 (q, 1H, J = 1.0 Hz, 3-H), 2.44 (d, 1H, J = 1.0 Hz, 4-Me), 2.41 (d, 1H, J = 0.9 Hz, 6-Me), 2.37 (q, 1H, J = 0.9 Hz, 8-Me or 9-Me), 2.34 (q, 1H, J = 0.9 Hz, 8-Me or 9-Me); ^{13}C NMR (DMSO-d_6) δ 162.05 (m, C-2), 154.32 (m, C-6a), 151.01 (broad q, J = 6.0 Hz, C-4), 150.03 (q, J = 7.2 Hz, C-8), 132.02 (d, J = 9.0 Hz, C-9b), 121.26 (dq, J = 160.0, 5.2 Hz, C-5), 119.01 (dq, J = 164.0, 5.0 Hz, C-3), 116.33 (q, J = 6.2 Hz, C-6), 116.02 (m, C-4a o C-9a), 115.62 (m, C-4a o C-9a), 110.50 (q, J = 4.8 Hz, C-9), 20.42 (qd, J = 127.8, 5.7 Hz, 4-Me), 15.49 (qd, J = 123.0, 4.8 Hz, 6-Me), 12.20 (q, J = 128.7 Hz, 8-Me), 10.46 (q, J = 129.7 Hz, 9-Me); MS (EI) m/z 241 (M^+); anal. ($C_{15}H_{15}NO_2$): C, H, N.

Compound 3f. Mp 298 °C (MeOH); ^{1}H NMR (DMSO-d_6) δ 11.49 (broad s, 1H, –NH), 7.44 (s, 1H, 5-H), 6.67 (t, 1H, J = 4.4 Hz, 8-CH_2OH), 6.36 (q, 1H, J = 1.2 Hz, 3-H), 4.77 (d, 2H, J = 4.4 Hz, 8-CH_2OH), 2.48 (m, 9H, 4-Me, 8-Me and 9-Me); ^{13}C NMR (DMSO-d_6) δ 162.10 (m, C-2), 154.56 (m, C-6a), 150.74 (qd, J = 6.7, 4.0 Hz, C-4), 149.73 (t, J = 5.2 Hz, C-8), 131.62 (d, J = 8.6 Hz, C-9b), 121.65 (dq, J = 159.7, 5.2 Hz, C-5), 119.18 (dq, J = 164.0, 4.3 Hz, C-3), 116.14 (q, J = 5.7 Hz, C-6), 116.05 (m, C-4a o C-9a), 115.60 (m, C-4a o C-9a), 115.52 (q, J = 3.8 Hz, C-9), 55.26 (td, J = 143.1, 6.6 Hz, 8-CH_2), 20.30 (qd, J = 127.8, 5.7 Hz, 4-Me), 15.49 (qd, J = 127.8, 4.8 Hz, 6-Me), 12.76 (q, J = 128.7 Hz, 9-Me); MS (EI) m/z 257 (M^+); anal. ($C_{15}H_{15}NO_3$): C, H, N.

8-Hydroxymethyl-4,6,9-trimethylfuro[2,3-*h*]quinolin-2(1*H*)-one (3f) from 3e. Compound **3e** (0.5 g, 2.5 mmol) was dissolved in concd. H_2SO_4 (10 mL) and the solution was heated at 50 °C until **3e** disappeared (3 h). The mixture was poured into cold water (100 mL), neutralized with 5% $NaHCO_3$ and extracted with AcOEt (3 × 100 mL). The organic phase was dried (Na_2SO_4), evaporated under reduced pressure and the residue purified by column chromatography to give **3f** (0.05 g, 10%), with mp, NMR and MS data as above reported.

Biology

8-MOP was obtained from Chinoin (Milan, Italy) and calf thymus DNA from Sigma-Chemie (Deisenhofen, Germany). The test compounds were dissolved in dimethyl sulfoxide (DMSO; 4.5 mM) and the solutions 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 solvent concentration never exceeding 0.5%. The same amount of DMSO was added to untreated controls. Every experiment was carried out at least three times.

Animals and tumor line

All procedures involving animals and their care were conducted in conformity with the institutional guidelines, that were in compliance with National and European Economic Community Council Directives. Ehrlich cells, Lettrè strains from Heidelberg, were routinely transferred by intraperitoneous injection (2×10^6 cells per mouse) into Swiss mice (LD_{50} after 30 days 5×10^2 cells). For the experiments mice at 8–10 weeks of age and at a mean body weight of 23 ± 2 g were used. Tumor cells were withdrawn from donor mice as a pure, highly concentrated, single cell suspension. For the experiments, they were collected by centrifugation and suspended in PBS.

UVA irradiation

Cell suspensions containing the test compound were incubated at room temperature for 15 min in the dark, put into Petri dishes (5 cm in diameter, 3 mL) and exposed to UVA light. UVA exposure were performed with a Philips HPW 125 lamp, provided with a built-in Philips filter. The emission spectrum was in the range 320–400 nm, with a maximum, over 90% of the total, at 365 nm. Irradiation intensity was determined on a UV radiometer (model 97507, Cole-Parmer Instrument Co., Niles, IL) at 5.5×10^{-3} KJ s^{-1} m^{-2}.

DNA synthesis in Ehrlich cells

DNA synthesis was assayed in Ehrlich ascites tumor cells (Lettrè strain) as already described.[11] Just after UVA irradiation (cell density: 2×10^7 cells mL^{-1} in PBS), the samples (10^6 cells in 0.5 mL PBS) were incubated for

30 min at 37° in the presence of 40 kBq mL^{-1} of [^{3}H]thymidine (4.77 TBq mM^{-1}; Amersham International Inc., UK). The acid-insoluble fraction was precipitated by adding ice-cold 5% 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. The results were calculated as the percentage of radioactivity incorporated into the DNA with respect to untreated control cells (approximately 3–6 kBq). Filtrations were carried out with a Sample Manifold apparatus (Millipore Corp., Bedford, USA). The filters were dried and counted. Data are expressed as ID_{50}, that is the UVA dose (KJ m^{-2}) inducing 50% inhibition of the macromolecular synthesis in the presence of 20 μM drug concentration. Data were computed by probit analysis.

Clonal growth of HeLa cells

HeLa cells were grown in nutrient mixture F-12Ham medium (Sigma Chemical Co, St. Louis, MO, USA), containing 10% fetal calf serum, and supplemented with antibiotics. Cell growth was accomplished at 37 °C in a 5% carbon dioxide atmosphere.

HeLa cells ($1.5–2\times10^5$) were seeded in Petri dishes in growth medium (4 mL). After 24 h, the medium was replaced with a fresh one containing the test compound. The cells were incubated for 15 min at 37 °C in the dark and then exposed to UVA light. Aliquots of 100 cells were seeded in the same medium, incubated for 7 days and then the 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 the formed colonies and the number of the cells seeded, was then calculated. The plating efficiency was about 90%. Data are expressed as ID_{50}, that is the UVA dose (KJ m^{-2}) inducing 50% inhibition of the clonal growth in the presence of 5 μM drug concentration. Calculations were accomplished by probit analysis.

Experiments with T2 phage

The host bacteria (*E. coli* B48) were grown in cultures prepared in brain-heart infusion (Difco Laboratories, Detroit, MI, USA) at 37 °C, collected in log phase, suspended in $MgSO_4$ (2 mM) at a density of 10^9 cells mL^{-1}, and then infected with T2 phage at a multiplicity of 1. The culture was then incubated at 37 °C for 3 h. Phage titers were determined using the standard bilayer method[22] and the same *E. coli* strain. Phage suspensions were diluted to 10^{10} viral particles/mL with $MgSO_4$ (2 mL) containing the test compound. Aliquots (5 mL) of these virus suspensions were poured into Petri dishes (5 cm in diameter), incubated at 37 °C for 25 min in the dark and then exposed to UVA light. After irradiation, the viral suspensions were further diluted with the same medium and the numbers of plaque-forming units/mL were scored.

Mutagenesis tests

The strain used was *E. coli* TM9 (*WP2*, *uvrA*, *R46*) carrying a nonsense mutation in the *trpE* gene which is reverted by UV light and by most base pair substitution mutagens[23] and therefore by formation of C_4-cycloadducts of furocoumarins.[4] Bacteria were grown overnight in a minimal Davis–Mingioli salt glucose medium supplemented with tryptophan (20 mg L^{-1}). *E. coli* cells were washed and then suspended in PBS (pH 7.0) containing the test compound (20 μM) at a density of 10^8 cells mL^{-1}. Bacteria were irradiated with UVA. 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} 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 plateled on Davis–Mingioli minimal medium supplemented with tryptophan. The plates were incubated for 24 h at 37 °C in the dark, and the colonies were then 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 result the number of revertant colonies per million survivors observed in controls (0.03 ± 0.01).

Skin phototoxicity

Skin phototoxicity was tested applied on depilated albino guinea-pigs (outbred Dunkin–Hartley strain) as already described.[24] Compounds were applied topically as 0.5% methanolic solutions up to 5×10^{-2} mg cm^{-2}. The animals were kept in the dark for 15 min and then exposed to UVA light (20 kJ m^{-2}). The animals were observed for 7 days.

Spectrophotometric determinations

A **3b** solution (8.2×10^{-5} M; EtOH/water, 1:1) was exposed to increasing UVA light doses (0, 0.8, 1.6, 3.2, 6.4, 12.8 and 25.6 kJ m^{-2}) and the UV spectrum was then recorded on a Kontron UVIKON-930 UV-Visible spectrophotometer.

Radiochemical determinations

Filters from DNA 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 and neutral elution were counted using Ultima Gold XR (Packard Instruments, Meriden, CT, USA) and a Packard Tri-Carb 1900TR spectrometer. Counting was accomplished automatically on the basis of quenching curves obtained using [^{14}C] and [^{3}H] radioactivity standards.

Photoreaction with DNA in vitro

Samples (3 mL) of an aqueous solution of DNA (2.3 mM) containing 2 mM NaCl, 1 mM EDTA and compound **3b** (4 μM), were exposed to UVA light (10 kJ m^{-2}), made up to 1 M in NaCl and treated with two volumes of ethanol. The precipitated DNA, washed

with 80% ethanol, was dissolved in 3 mL of water. The fluorescence was then determined on a Perkin Elmer model LS-5 spectrophotofluorimeter.

Detection of DNA damage

DNA damage was detected by alkaline or neutral elution according to Kohn;[17] each experiment was carried out using an internal standard, that is untreated cells labeled with [^{3}H]thymidine and submitted only to a well defined dose of gamma rays, while treated cells were labeled with [^{14}C]thymidine. HeLa cells in exponential growth were labeled by overnight incubation in the presence of 7.4 KBq mL^{-1} [^{3}H]thymidine or 3.7 KBq mL^{-1} [^{14}C]thymidine. The radioactive medium was removed and replaced by a fresh one containing the test compound for [^{14}C]cells, and by a fresh one containing only 0.5% DMSO for [^{3}H]cells. The cells were exposed to UVA, washed, and submitted to alkaline elution.

Detection of inter-strand cross-links (ISC). About $0.5–1.0\times10^6$ of treated [^{14}C]cells were mixed with equal amounts of standard [^{3}H]cells, the mixture was cooled in ice, and exposed to 6 Gy of gamma rays. The mixture was deposited on a polycarbonate filter (pores 2 μm in diameter, Nucleopore Corp., Pleasanton, CA, USA) in a Swinnex-25 filter holder (Millipore Corp., Bedford, MA, USA) and immediately lysed with 2% SDS, 0.1 M glycine, 0.025 M Na_2EDTA, pH 10, (5 mL). The solution was then allowed to flow out by gravity. The same solution (2 mL) containing 0.5 mg L^{-1} of proteinase K (Sigma Chemical Co., St. Louis, MO, USA) was gently poured on to the filter, followed by 40 mL of the eluting solution (1 M tetrapropylammonium hydroxide, 0.02 M EDTA, 0.1% SDS; pH 12.1). Elution was carried out with a Gilson Minipuls peristaltic pump, at a flow rate of 0.03–0.04 mL min^{-1}. The radioactivity of both isotopes was determined in the fractions collected with a Gilson fraction collector (approximately, 3.5 mL per fraction).

Detection of DNA-protein cross-links (DPC). The procedure was the same reported for ISC, but the gamma ray dose delivered to the mixed cell suspensions was increased to 30 Gy; polyvinyl chloride filters (pores 5 μm in diameter; Nucleopore Corp., Bedford, MA, USA) were employed and treatment with proteinase K was omitted. All calculations were performed according to Kohn[17] and as already described.[14] 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 2.5 Gy min^{-1}, as determined by Fricke solution.

Detection of DNA double-strand breaks (DSB). DSB were detected by neutral elution. HeLa cells were labeled as before by 7.4 KBq mL^{-1} [^{3}H]thymidine and then submitted to sensitization with the test compound as described. The cells were washed with PBS and then submitted to the elution procedure as described for ISC detection, eluting with a different solution (1 M tetrapropylammonium hydroxide, 0.02 M EDTA, 0.1% SDS; pH 9.6). In these experiments no internal controls were used. The data obtained by neutral elution assay were computed as DNA fragmentation (F) on the basis of its radioactivity, as shown by the equation:

$$F = \frac{R_{\text{eluted}}}{R_{\text{eluted}} + R_{\text{filter}}}$$

where R_{eluted} is the total eluted radioactivity and R_{filter} is that retained on the filter.

The results are expressed as the percentage of DNA fragmentation in comparison with the controls according to the formula:

$$\text{Percent of DNA fragmentation} = \frac{F - F_0}{1 - F_0} \times 100$$

where F is the DNA fragmentation observed in the treated sample and F_0 is that scored in the controls.[25]

Experiments with the double irradiation protocol

The double irradiation procedure was carried out by exposing HeLa cells to a small UVA dose in the presence of the compound to be tested. The drug-containing medium was then carefully discharged and the cells washed twice with aliquots (15 mL) of medium free of drug and serum. A third portion of fresh medium was then added and the cells were further exposed to UVA light.[14]

Experiments in mice

Ehrlich cells were irradiated in the presence of the test compound and then injected intraperitoneously (10^6 cells per mouse) into healthy mice. The animals were then observed for 90 days, scoring mortality due to tumor growth.

In the experiments concerning protection against the tumor, Ehrlich cells were irradiated in the presence of test compound in experimental conditions sufficient to inhibit their ability to transmit the tumor; and they were then injected into healthy mice. The challenge was performed by injecting an established number of viable Ehrlich cells on the selected day, according to the experimental protocol. Animals were observed for 90 days.

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