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Photobiological Properties of 1′-Thieno-4,6,4′-trimethylangelicin

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ABSTRACT

Some photobiological properties of 1′-thieno-4,6,4′-trimethylangelicin (TTMA), a new isoster of 4,6,4′-trimethylangelicin (TMA) were studied in comparison with the parent compound. The TTMA absorbs UVA light and photobinds *in vitro* to DNA more efficiently than TMA; however, in Ehrlich cells *in vivo* TTMA linked to DNA to a lesser extent than the parent compound. In general, the formation of damage into DNA is in line with this last result: In fact, TTMA and TMA form equivalent amounts of interstrand cross-links (ISC) both *in vitro* in linearized PM2 DNA and *in vivo* in HeLa cells. In this system TTMA induces DNA–protein cross-links (DPC) more efficiently than TMA; on the contrary, no significant amounts of single-strand breaks were detected with both compounds. The antiproliferative activity of TTMA is consistent with these results, being only slightly more pronounced than that of TMA. Experiments carried out using double irradiation demonstrated that these drugs are capable of inducing antiproliferative effects by biphotonic reactions, including the formation of both ISC and DPC. Thus, replacement of the oxygen atom by a sulfur increases the UV absorption of the drug and its capacity to photobind to DNA *in vitro* but does not yield a comparable enhancement of its photosensitizing properties *in vivo*; this might be due to various reasons, for instance to an increase in the lipophilic character that could modify the behavior *in vivo*.

INTRODUCTION

Furocoumarins (psoralens and angelicins) are active drugs with strong and interesting photobiological properties (1). Some of them have been successfully employed for several years in photomedicine as PUVA† therapy (psoralen plus UVA) in the treatment of various skin diseases (2); recently they have also been used in photopheresis, the extracorporeal treatment of lymphocytes to obtain selective immune modulation in T-cell lymphoma therapy, for the prevention of rejection in organ transplantation and as treatment for some autoimmune diseases (3,4). However, the compound generally used, 8-methoxypsoralen (8-MOP), has various unpleasant side effects, in particular, a strong genotoxicity that is associated with the formation of covalent adducts in DNA and mainly with interstrand cross-links (ISC) (5–7). Thus, several monofunctional derivatives have been prepared and studied (8,9), in particular angelicin derivatives; actually, the geometry of their angular molecular structure prevents the formation of bifunctional adducts between two pyrimidine bases placed on opposite DNA strands, *i.e.* ISC (9). Nevertheless, the most effective and promising angelicin derivative, 4,6,4′-trimethylangelicin (TMA) (9,10), appeared to be capable of inducing DNA ISC, even if to a lower extent when compared with 8-MOP, as observed *in vitro* (11) and *in vivo* (12).

More recently, to develop new derivatives with better features than 8-MOP, various authors have focused on the insertion of a heteroatom into the furocoumarin skeleton, thus obtaining furocoumarin isosters, such as, for example, some azapsoralens (13–15) and various sulfur and selenium derivatives (16–19). Angelicin isosters were also studied, in particular, some methylfuroquinolinones, in which a nitrogen atom replaces the oxygen at the pyronic ring (20–22). In addition some 4-amino-3-chloroangelicin derivatives carrying a sulfur atom substituting for the oxygen at the furan ring were also prepared (23). More recently some methyl derivatives of thienoangelicins have been synthesized (24); among them is 1′-thieno-4,6,4′-trimethylangelicin (TTMA),

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†*Abbreviations:* BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; DPC, DNA–protein cross-links; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EtBr, ethidium bromide; ISC, interstrand cross-links; 8-MOP, 8-methoxypsoralen; PBS, phosphate-buffered saline; PUVA, psoralen plus UVA; SSB, single-strand breaks; TAE, 40 m*M* Tris-sodium acetate, pH 8.2, 1 m*M* EDTA; TE, 40 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA; TMA, 4,6,4′-trimethylangelicin; TTMA, 1′-thieno-4,6,4′-trimethylangelicin.

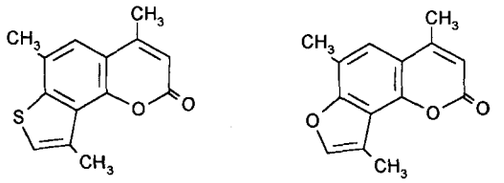

Figure 1. Molecular structure of 1′-thieno-4,6,4′-trimethylangelicin (TTMA) and 4,6,4′-trimethylangelicin (TMA).

a sulfur isoster of TMA. Considering the high activity and the particular features of TMA (9,10), we studied some of the photochemical and photobiological properties of TTMA in comparison with TMA, assumed as a reference compound.

MATERIALS AND METHODS

Chemicals

Both TTMA (24) and TMA (25) were prepared by chemical synthesis. The molecular structures of these compounds are reported in Fig. 1. In some experiments, 8-MOP (from Chinoin, Milano, Italy) was also used. Compounds were dissolved in dimethyl sulfoxide (DMSO; 4.5 m*M*) and the solutions were kept at −20°C in the dark. Just before the experiment, a calculated amount of drug solution was added in the dark to phosphate-buffered saline (PBS) or to the growth medium containing cells to a final solvent concentration of 0.5%. The DMSO, which had no discernible effect on cells at this concentration (data not shown), was added also to the control cells in every experiment. ^{3}H-thymidine (4.77 TBq mM^{-1}), ^{14}C-thymidine (2.2 GBq mM^{-1}), labeled ^{3}H-TTMA (0.39 Bq M^{-1}) and ^{3}H-TMA (2.97 TBq M^{-1}) and PstI were all from Amersham International Inc., UK. Proteinase K and PM2 DNA were purchased from Boehringer Mannheim GmbH, Germany. The DNA from salmon testes, a 1 *M* aqueous solution of tetrapropylammonium hydroxide, bovine serum albumin (BSA), dithiothreitol (DTT), bromophenol blue and ethidium bromide (EtBr) were obtained from Sigma-Chemie, Deisenhofen, Germany.

UVA irradiations

The DNA solutions were irradiated in test tubes immersed in a thermostatically controlled cell at 20°C. Cell suspensions containing the drug to be studied were incubated at room temperature for 15 min in the dark and then put into petri dishes (5 cm diameter; 3 mL), placed on an ice bath and then exposed to UVA light. Samples for alkaline elution were kept on ice also during the successive treatments (*e.g.* gamma irradiation), until the lysis step. Exposures to UVA were performed with Philips HPW 125 lamps, provided with a built-in Philips filter; their emission was in the range of 320–400 nm, with a maximum, over 90% of the total, at 365 nm. The irradiation intensity, determined by a radiometer (mod. 97503, Cole-Parmer Instrument Co., Niles, IL, USA) was 0.9×10^{-6} W m^{-2}.

UV determinations

The UV spectra were measured in absolute ethanol using a Kontron UVIKON-930 UV–visible spectrophotometer.

DNA photobinding *in vitro*

Aqueous solutions (2.3 m*M*) of DNA from salmon testes containing 2 m*M* NaCl, 1 m*M* ethylenediaminetetraacetic acid (EDTA) and ^{3}H-TTMA or ^{3}H-TMA (30 μ*M*, checked by radiochemical measurements) were exposed to increasing UVA doses; then solid NaCl was added up to 2 *M*, followed by 2 volumes of ethanol. The precipitated DNA, collected by centrifugation, was washed with 80% ethanol and dissolved in the initial volume of water.

Detection of ISC *in vitro*

Supercoiled circular DNA of PM2 bacteriophage (4 μg) was linearized by incubating at 37°C for 2 h with PstI (18 U μL^{-1}) in a 100 μL solution containing 10 m*M* NaCl, 50 m*M* Tris-HCl, pH 7.5, 10 m*M* $MgCl_2$, 1 m*M* DTT and 10 μg BSA. The solution was filtered in Microcon 100 tubes (Amicon) by centrifuging at 500 ***g*** for 5 min. The membrane was washed by adding 100 μL of TE (40 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA) and the solution was stirred and centrifuged at 500 ***g*** for an additional 5 min. The solution over the membrane was then recovered by inverting the filter and centrifuging at 1000 ***g*** for 3 min. The remaining solution was diluted by adding TE to obtain a DNA concentration of 80 ng μL^{-1}. The solution was then stirred at 4°C until use.

To detect the formation of ISC, 2 μL of PM2-linearized DNA for each sample was added to 2 μL of a solution (4× in DMSO) of the drug to be tested (TMA, TTMA or 8-MOP) at the appropriate concentration so that the final molar ratio drug/base pairs was 2; 4 μL TE was then added to each sample so that the final volume was 8 μL.

Duplicate samples were incubated at room temperature in the dark for 15 min and then exposed to UVA light. As a control, 2 μL of linearized PM2 DNA (80 ng μL^{-1}) was added to 2 μL DMSO and to 4 μL TE, incubated as described previously but not irradiated. Then, 2 μL aliquots of a solution containing 1.5 *M* sodium acetate, 100 m*M* EDTA were added to the samples that were then stirred and centrifuged at 10 000 ***g*** for 1 min. The DNA was precipitated with three volumes (30 μL) 95% ethanol and samples were held at −80°C for at least 1 h; DNA was recovered by centrifugation at 2000 ***g*** for 20 min at 4°C. Precipitated DNA was washed with the same solvent and then suspended in 7 μL TAE (40 m*M* Tris-sodium acetate, pH 8.2, 1 m*M* EDTA) containing 0.04% bromophenol blue, 10% glycerol and 30% DMSO. The DNA was denatured by heating at 90°C for 2 min and quickly cooling on ice.

The samples were analyzed by agarose gel (0.7%) electrophoresis at 50 V in TAE containing 0.5 μg mL^{-1} EtBr. The gel was photographed with a Polaroid camera placed over a UV transilluminator TM36 (UVP Inc., San Gabriel, CA, USA).

Experiments with Ehrlich cells

DNA photobinding in vivo. Ehrlich cells (Lettrè strain, 2×10^{7} cells mL^{-1}) were exposed to UVA light in the presence of ^{3}H-TTMA or ^{3}H-TMA (6 μ*M*) and then the cells (10^{6} per sample) were washed with PBS and lysed in hot ethanol. The precipitated material was extracted with a 10% NaCl solution (90°C); after low-speed centrifugation, DNA was precipitated from the supernatant by ethanol, collected, washed with 80% ethanol and dissolved in 1 mL of water. The DNA content (26) and radioactivity were determined. The data are the means of at least triplicate determinations.

DNA synthesis in Ehrlich cells. Synthesis of DNA was assayed in Ehrlich cells as already described (21); briefly, just after UVA irradiation (cell density: 2×10^{7} cells mL^{-1}), 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} of ^{3}H-thymidine. The acid-insoluble fraction was precipitated by adding ice-cold 5% trichloroacetic acid and then filtering 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 percent radioactivity incorporated into the DNA of untreated control cells (approximately 3–6 kBq). Filtrations were carried out with a Sample Manifold apparatus (Millipore Corporation, Bedford, USA). Every experiment, performed using duplicate samples per point, was carried out at least three times.

Experiments with human cells cultivated *in vitro*

The HeLa cells were grown in nutrient mixture F-12 Ham (Sigma Chemical Co., St. Louis, MO, USA) medium, containing 5% fetal calf serum (increased to 10% in the clonal growth experiments). In both cases, media were supplemented with antibiotics and cell growth was accomplished at 37°C in a 5% carbon dioxide atmosphere. Every experiment was carried out at least three times.

Clonal growth. Aliquots of $1.5–2 \times 10^{5}$ HeLa cells were seeded in petri dishes in growth medium (4 mL). After 24 h the medium

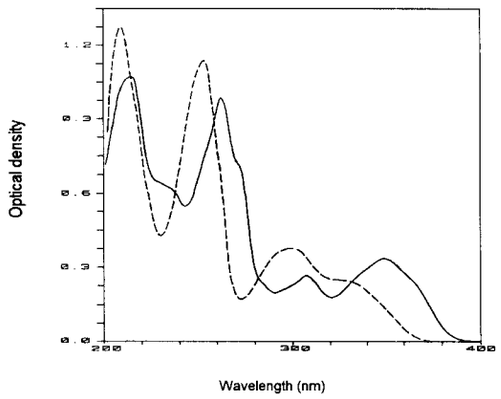

Figure 2. Ultraviolet spectra of TTMA (4.83×10^{-5} *M*, solid line) and of TMA (4.64×10^{-5} *M*, dotted line) in absolute ethanol.

was replaced with a fresh one containing the compound to be studied, and then the cells were exposed to UVA light. Aliquots of 200 cells were seeded in growth medium, incubated for 7 days and then colonies were stained and counted, discarding those with fewer than 50 cells. The efficiency of the clonal growth, *i.e.* the ratio between the number of colonies formed and the number of cells seeded, was then calculated. Plating efficiency was about 80%.

Detection of DNA damage. Damage to DNA was detected by alkaline elution performed according to Kohn (27); each experiment was carried out using an internal standard, *i.e.* 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.

The HeLa cells in exponential growth were labeled by overnight incubation in the presence of ^{3}H-thymidine (7.4 kBq mL^{-1}) or ^{14}C-thymidine (3.7 kBq mL^{-1}). For the experiments, the radioactive medium was removed; for ^{14}C-labeled cells medium was replaced with one containing the compound to be studied, while for ^{3}H-labeled cells, medium contained only 0.5% DMSO. The cells were exposed to UVA, washed and submitted to alkaline elution.

In the single-strand break (SSB) determinations, about $0.5–1.0 \times 10^6$ of treated ^{14}C-labeled cells were mixed with equal amounts of ^{3}H-labeled 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, USA) in a Swinnex-25 filter holder (Millipore Corp., Bedford, MA, USA) and immediately lysed with 2% sodium dodecylsulfate, 0.1 *M* glycine, 0.025 *M* Na_2EDTA, pH 10 (5 mL); after this, the solution was allowed to flow out by gravity. Then 2 mL of the same solution containing 0.5 mg L^{-1} of proteinase K (Sigma Chemical Co.) was gently poured on the filter, followed by 40 mL of the eluting solution (tetrapropylammonium hydroxide–EDTA–0.1% sodium dodecylsulfate, pH 12.1). Elution was carried out with a Gilson Minipuls peristaltic pump, at a flow of 0.03–0.04 mL min^{-1}. Fractions were collected with a Gilson fraction collector (~3.5 mL per fraction) and the radioactivity of both isotopes was then determined.

In the ISC determinations, $\sim 0.5–1.0 \times 10^6$ of treated ^{14}C-labeled cells were mixed with equal amounts of standard ^{3}H-labeled cells, then the mixture was cooled on ice and exposed to 6 Gy of gamma rays. Elutions were then performed as above described for SSB determinations.

To detect DPC, the procedure was the same reported for ISC, but the gamma ray dose was increased to 30 Gy; moreover, polyvinyl chloride filters (pores 5 μm in diameter; Nucleopore Corp.) and a lysis solution containing 0.1% sodium *N*-lauroylsarcosine, 0.04 *M* Na_2EDTA, pH 10 was employed, while the treatment with proteinase K was omitted.

All calculations were performed according to Kohn (27), as already described (21).

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.

Double-irradiation protocol. The double-irradiation protocol was accomplished by exposing cells to a small UVA dose in the presence of the compound to be examined. The furocoumarin-containing medium was carefully discharged and cells were washed two times with 15 mL aliquots of medium free of drug and of serum; a third portion of fresh medium was added and the cells were further irradiated (21).

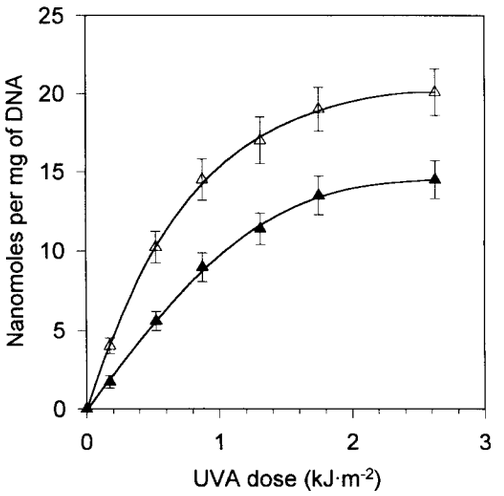

Figure 3. Photobinding to salmon testes DNA *in vitro*: both ^{3}H-TTMA (△) and ^{3}H-TMA (▲) were used at a 30 μ*M* concentration. The bars represent the standard deviation.

Radiochemical determinations. Filters were counted by using a toluene-based scintillation fluid (PPO 5 g, dimethyl-POPOP 0.25 g, toluene up to 1 L of solution). For DNA solutions and the fractions from alkaline elution, Ultima Gold XR (Packard Instruments, Meriden, CT, USA) was used. All determinations were carried out in a Packard Tri-Carb 1900TR spectrometer. Double-isotope counting was accomplished automatically on the bases of quenching curves obtained using ^{3}H- and ^{14}C-radioactivity standards.

RESULTS

Spectrophotometric features and photostability

Figure 2 shows the UV spectra of TTMA in comparison with TMA. In the UVA wavelength range, TTMA absorbs light much more efficiently than TMA. In fact, its extinction coefficient at 365 nm, the maximum of the emission of the lamps used, is about 5400, in comparison with 600 for TMA.

We have also preliminarily studied the photostability of TTMA under UVA irradiation; at the highest doses used in this work, we observed only some modifications of the UV spectrum. In particular we observed a decrease of 12% and 17% of the optical density after 5 and 10 kJ m^{-2} at the 307 nm peak. With TMA, assayed under similar experimental conditions, we observed a reduction of 6 and 14%, respectively.

DNA damage *in vitro*

Photobinding to salmon testes DNA. Aqueous solutions of salmon testes DNA were exposed to increasing UVA doses in the presence of ^{3}H-TTMA or of ^{3}H-TMA. The results are reported in Fig. 3. The TTMA showed strong DNA photobinding capacity, higher than that exhibited by TMA.

ISC formation in PM2 DNA. Aqueous solutions of linearized PM2 DNA were exposed to UVA light in the presence of TTMA or of TMA (two drug molecules per DNA

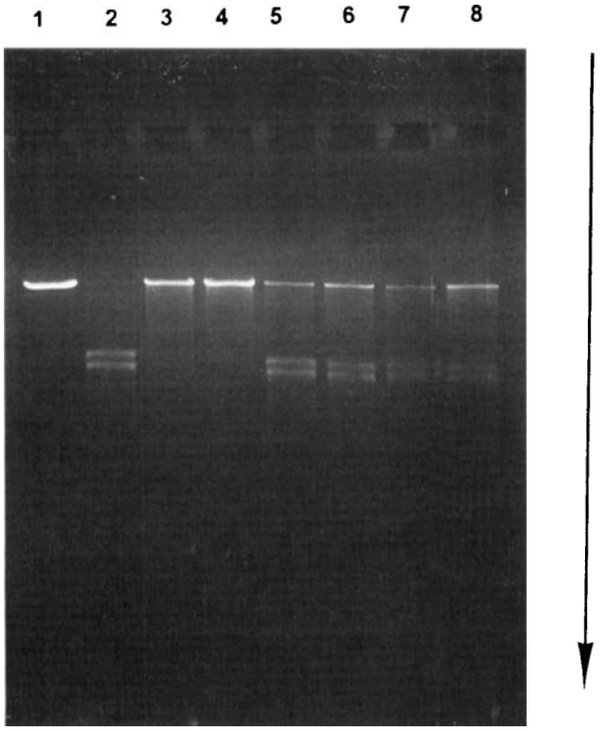

Figure 4. Interstrand cross-links formed *in vitro* in linearized PM2 DNA by TTMA sensitization (two drug molecules per base pair) detected by electrophoresis. For comparison, TMA and 8-MOP were also studied in the same experimental conditions. The lanes represent: 1, double-stranded DNA; 2, denatured single-stranded DNA; DNA irradiated in the presence of: 3 and 4, 8-MOP; 5 and 6, TMA; 7 and 8, TTMA. Lanes 3, 5 and 7 represent DNA exposed to 1.5 kJ m^{-2}; lanes 4, 6 and 8, DNA exposed to 3 kJ m^{-2}. The arrow indicates direction of electrophoretic migration.

base pair). As positive controls, similar experiments were carried out with 8-MOP; UVA irradiation alone until 10 kJ m^{-2} did not modify the electrophoretic migration of DNA (data not shown). The DNA samples were heat denatured and then submitted to electrophoresis. The results are reported in Fig. 4. Significantly more DNA irradiated in the presence of TTMA migrated as the level of double-stranded (cross-linked) DNA and its amounts increased with UVA dose. The TMA appeared to be almost as effective as TTMA. These data clearly showed that linearized PM2 DNA had been cross-linked by both drugs. As expected, 8-MOP, used as a positive control, induced high levels of ISC.

DNA damage *in vivo*

Photobinding to Ehrlich cell DNA. Ehrlich cells were exposed to increasing doses of UVA light in the presence of ^{3}H-TTMA or ^{3}H-TMA (6 μM) and then the cellular DNA was extracted and its specific activity was determined. The results are reported in Fig. 5. The results are very different from those obtained with salmon testes DNA *in vitro*; *in vivo* TTMA appeared to be less effective than TMA, even though the differences are modest.

Formation of breaks and covalent cross-links in HeLa cell DNA. The HeLa cells were irradiated with UVA light in the presence of TTMA or of TMA and then submitted to alkaline elution. The data are reported in Fig. 6. Panel A shows the results obtained by studying the formation of SSB. Both cell samples exposed to UVA light (5 kJ m^{-2}) in the presence of TTMA or of TMA (20 μM) generated elution profiles very close to that of untreated controls; therefore, both compounds induced very low levels of SSB in DNA ($\leq$0.14 breaks per million nucleotides).

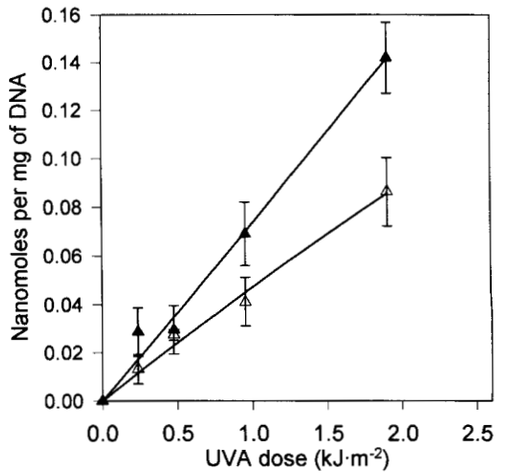

Figure 5. Photobinding to DNA of Ehrlich cells *in vivo*: ^{3}H-TTMA (△) and ^{3}H-TMA (▲) were used at a 6 μM concentration. The bars represent the standard deviation.

The data related to the detection of ISC are reported in panel B. Both TTMA and TMA (20 μM plus 5 kJ m^{-2} of UVA) induced apparent DNA retention in comparison with the profile generated by cells treated only with 6 Gy of gamma rays. This is a clear indication of ISC formation. In this reaction, TTMA appeared to be only slightly more active than TMA; the cross-linking coefficient, a parameter proportional to the number of ISC (27), is 0.067 ± 0.01 for TTMA and 0.06 ± 0.009 for TMA. In parallel experiments, 8-MOP, used as a positive control, gave a value of 0.46 ± 0.07 (data not shown). The data related to TMA are consistent with those already obtained (12) in which TMA appeared to be capable of inducing about 15% of the ISC formed by 8-MOP.

Panel C shows the results of the experiments carried out for DNA–protein cross-link (DPC) detection. Using the same conditions as for ISC, TTMA induced a marked retention of DNA, with significant evidence of DPC formation; TMA seemed to be less active. The estimated DPC frequencies per million nucleotides were 0.36 ± 0.03 lesions for TTMA and 0.19 ± 0.015 for TMA. Under comparable conditions, 8-MOP was much more effective (21).

Antiproliferative activity

Figure 7 shows the results obtained by studying the antiproliferative activity of TTMA (6 μM) using two different tests, the inhibition of DNA synthesis in Ehrlich cells and of clonal growth in HeLa cells. The TTMA appeared to be capable of inducing a strong antiproliferative effect; however, the curves generated by TTMA in both tests show small differences when compared with that obtained using TMA.

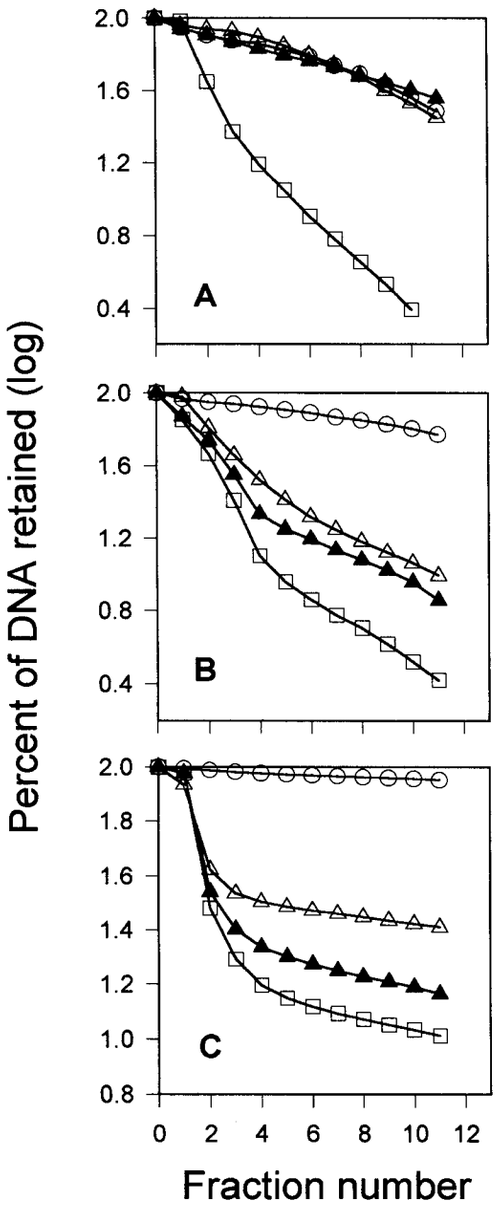

Figure 6. Damage to DNA induced by TTMA and TMA detected by alkaline elution. Both compounds were used at a 20 μM concentration plus 5 kJ m^{-2}. A: Single-strand breaks (SSB): control cells, zero Gy, ○; control cells, 6 Gy, □; cells sensitized by TTMA, zero Gy, △; cells sensitized by TMA, zero Gy, ▲. B: Interstrand cross-links (ISC): control cells, zero Gy, ○; control cells, 6 Gy, □; cells sensitized by TTMA, 6 Gy, △; cells sensitized by TMA, 6 Gy, ▲. C: DNA–protein cross-links (DPC): control cells, zero Gy, ○; control cells, 30 Gy, □; cells sensitized by TTMA, 30 Gy, △; cells sensitized by TMA, 30 Gy, ▲.

Experiments using the double-irradiation protocol

The activity of TTMA was also investigated using the double-irradiation method; the results are summarized in Table 1. To study the formation of DPC, HeLa cells were submitted to the first irradiation step (2.5 kJ m^{-2}) in the presence of TTMA (20 μM); the cells were washed to remove the unbound drug and then exposed again to UVA (3.3 kJ m^{-2}). The cells were then submitted to alkaline elution. Similar experiments were carried out also with TMA. Both compounds induced an apparent increase in DPC after the second irradiation step; TTMA appeared to be only slightly more active than TMA. Because HeLa cells are very sensitive, for the experiments on clonal growth capacity milder experimental conditions were used: 2 μM concentration plus 0.15 kJ m^{-2} for the first irradiation step. The second irradiation was carried out with two different UVA doses, 0.83 and 3.33 kJ m^{-2}. As expected, the second exposure to UVA light noticeably reduced the surviving fraction, with a clear relationship to the UVA dose. Even in these experiments, both compounds gave similar results, although TTMA appeared to be a little more active.

DISCUSSION

The TTMA, a new isoster of TMA, has some interesting features: it absorbs UVA light more efficiently than its parent compound and its capacity to photobinding to DNA from salmon testes *in vitro* appears to be higher than that for TMA. These results are consistent with the data already obtained for a psoralen isoster carrying a sulfur atom at the 1 position and a selenium atom at the 1′ position, which exhibited a DNA photobinding capacity about five times higher than the parent compound (19). Therefore, we predicted that TTMA might be more effective than TMA. However, after studying its antiproliferative activity by two different tests, inhibition of DNA synthesis in Ehrlich cells and clonal growth in HeLa cells, TTMA appeared to be only slightly more active than TMA.

To clarify this picture further, we studied the capacity of TTMA to damage DNA, *in vitro* and *in vivo*. First, we studied its photobinding capacity to DNA *in vivo* in Ehrlich ascites cells; in this system the amount of TTMA linked covalently to DNA was somewhat lower than that of TMA. Studying the formation of ISC *in vitro* in linearized PM2 DNA by electrophoresis, we observed that both TTMA and TMA formed comparable amounts of ISC.

Detecting ISC formation *in vivo* in HeLa cells by alkaline elution, we found that TTMA and TMA induced about 14–15% of the ISC of 8-MOP, a result consistent with that already observed for TMA (12). Moreover, in the same system we also observed that TTMA and TMA induced equivalent and low levels of SSB; to the contrary, TTMA appeared to be moderately more efficient than TMA in forming DPC, with a frequency of about 0.36 and 0.19 lesions per million nucleotides, respectively. We must remember that 8-MOP induces this lesion at much higher levels (21) and, contrary to TTMA and to TMA, forms SSB efficiently (21,28).

In conclusion, TTMA appears to be almost as active as the parent compound. This result is dependent upon its different behavior *in vitro* and *in vivo*. The insertion of a sulfur atom increases the lipophilic character of the molecule that can interfere seriously with the behavior of TTMA *in vivo*. On the other hand, similar results have been obtained by studying the TMA photobinding to DNA *in vivo* and *in vitro* (29) and this fact was attributed to its strong lipophilicity, which concentrates the compound into lipids, thus affecting its disposability at the level of DNA.

We also investigated the significance of the formation of

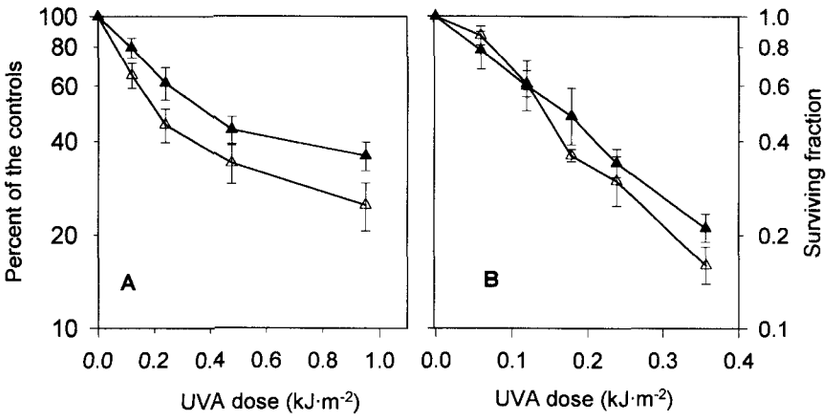

Figure 7. Antiproliferative activity of TTMA. A: Inhibition of DNA synthesis in Ehrlich cells. B: Inhibition of the clonal growth capacity of HeLa cells. Both compounds were assayed at a 6 μM concentration. The symbols are TTMA, △ and TMA, ▲. The bars represent the standard deviation.

bifunctional lesions into DNA (ISC and DPC) by TTMA. As is known, the formation of ISC requires the sequential absorption of two photons and can be efficiently studied by the so-called double-irradiation method (30,31); according to this protocol, the cells are irradiated with a small UVA dose in the presence of the compound to be studied, washed several times to remove the unbound drug and then exposed again to light. Under such conditions, during the second irradiation, some furan side monoadducts react further forming the covalent bridge between the two DNA strands. It is interesting to point out that in the absence of free sensitizer, no significant increase in the total number of DNA lesions formed takes place during the second irradiation but only the conversion of some monofunctional lesions into bifunctional ones. This allows a powerful estimate of the biological consequences of bifunctional damage (30,31).

Using this protocol in HeLa cells, we observed a marked decrease in survival after the second step with both drugs. This behavior, already observed with TMA (12), is consistent with a biphotonic reaction, for example, with ISC formation. However, after the second irradiation step, an increase in the DPC amount was also observed for both compounds. Using a tetramethylfuroquinolinone completely unable to form ISC and the same double-irradiation protocol (21), we obtained similar results; even in this case an increase in DPC number was detected after the second irradiation exposure. We suggested that DPC formation was responsible for this lethal effect. Therefore, we can also suggest that the lethality observed by the double irradiation carried out with TTMA and TMA may be related to the formation of both ISC and DPC.

Summarizing, in spite of its higher activity exhibited *in vitro,* TTMA exhibits photobiological behavior *in vivo* very close to that of TMA. Therefore, at least in this case, the substitution of an oxygen atom with a sulfur did not increase significantly the photosensitizing activity of the compound; moreover, this modification did not eliminate the ability of TMA to induce ISC, although it presents an angular molecular structure. Finally, on the bases of these results, the new sulfur and selenium isosters, until now tested only *in vitro* (16–19), must be carefully studied *in vivo* in whole mammalian cells to gain the full picture of their photobiological activity and of their photochemotherapeutic power.

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Table 1. Experiments carried out with the double-irradiation protocol*

	μM	UVA dose (kJ m^{-2})	DPC per 10^6 nucleotides (±SD)	μM	UVA dose (kJ m^{-2})	Surviving fraction (± SD)
TMA						
First step	20	2.5	0.1855 ± 0.04	2	0.15	0.47 ± 0.03
Second step	0	3.3	0.3750 ± 0.05	0	0.83	0.31 ± 0.016
				0	3.33	0.23 ± 0.017
TTMA						
First step	20	2.5	0.206 ± 0.03	2	0.15	0.33 ± 0.034
Second step	0	3.3	0.508 ± 0.04	0	0.83	0.18 ± 0.029
				0	3.33	0.15 ± 0.015

*HeLa cells were exposed to UVA light in the presence of the compound to be tested; the cells were washed to remove the unbound sensitizer and submitted to a second UVA irradiation. The DNA–protein cross-link frequencies and the surviving fraction after both the first and the second irradiation step were determined by alkaline elution and a clonogenic test, respectively.

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