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Photoreactivity of 5-Fluorouracil under UVB Light: Photolysis and Cytotoxicity Studies

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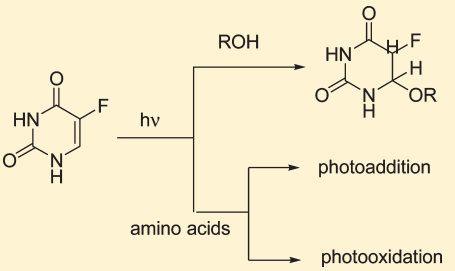
ABSTRACT: The photodegradation of the chemotherapeutic agent 5-fluorouracil (5-FU) under UVB light was studied both in aqueous and methanol solutions and in systemic and topical formulations. As monitored by HPLC, photodegradation in solution takes place in a concentration dependent manner; thus, the solution for parenteral administration (10^{-1} M) showed negligible loss of the active principle. On the contrary, the commercial cream containing 5% of 5-FU showed low stability under UVB exposure. When dissolved either in water or methanol, 5-FU yields two photoproducts which have been characterized as two isomers coming from the addition of the solvent to the 5,6 double bond of the drug. As a consequence, photomodified 5-FU loses its antiproliferative activity on HCT-15 and HeLa cells. MS analysis showed that photoaddition occurred with nucleophilic amino acids, such as cysteine and serine, while susceptible amino acids (cysteine and methionine) were oxidized. In fact, high production of the superoxide anion under UVB light as well as photooxidation of BSA suggests protein photodamage as a mechanism of photosensitization. Indeed, some phototoxicity was shown in experiments on NCTC keratinocytes and MCF-7 resistant cells irradiated with UVB light. The interactions with these biological targets may contribute to skin phototoxicity and photoallergy induced by 5-FU in vivo.



INTRODUCTION

The chemotherapeutic agent 5-fluorouracil (5-FU) is a pyrimidine analogue used in many dosage regimens alone or in adjuvant regimens as an antineoplastic drug given via I.V. push or bolus, or as continuous infusion principally for the treatment of colorectal cancers and pancreatic endocrine tumors. In addition, it is sometimes applied topically as a cream for treating actinic sun keratoses and some skin basal cell carcinomas.

As regards the interactions of this drug with light, 5-FU and its deoxyribonucleoside monophosphate are known to undergo photodegradation under UVC light.[1−3] In particular, Lozeron[1] showed that UVC irradiation of 5-FU gives rise to 5-fluoro-6-hydroxyhydrouracil as the main photoproduct. Therefore, the drug itself and its formulations are suggested to be protected from light.

Among the side effects, phototoxic and photoallergic reactions such as skin sensitivity to sun and hyperpigmentation on the face and palms of hands, are reported in treated patients inadvertently exposed to sunlight.[4,5] For this reason, it is suggested that topical or systemic 5-FU be administered during the winter months to prevent cutaneous side effects.

However, higher efficacy of 5-FU against actinic keratosis was found when affected areas were exposed to irradiation.[4] Moreover, since UVB light has demonstrated the ability to improve the efficacy of 5-FU in the treatment of certain solid tumors and advanced breast cancers, the drug has also been introduced as a radiosensitizer.[6,7]

Thus, the aim of this study was to investigate the UVA/UVB photodegradation of the compound, including isolation and identification of its main photoproducts both in solution and in commercial formulations. Furthermore, in order to obtain preliminary information about the biological consequences of 5-FU photolysis, the cytotoxic activity before and after irradiation was studied on sensitive tumor cell lines (HeLa and HCT-15). Phototoxicity was also tested, in particular on NCTC-2544 keratinocytes and 3T3 fibroblasts. The photoallergic potential was also evaluated through photosensitization reactions with model amino acids and proteins.

Finally, experiments with 5-FU resistant MCF-7 human breast cancer cells were performed in order to assess the antiproliferative effect of 5-FU in combination with UVB light.

MATERIALS AND METHODS

Caution: *5-FU is hazardous and should be handled carefully; the operator must protect himself against UVB radiation.*

Photolysis Experiments. Solid 5-FU (Serva Feinbiochemica GMBH & CO., Heidelberg, Germany) dissolved (10^{-5} M) in water or methanol and 5-FU for injection (Teva Italia S.r.l., Milano, Italy, containing 5 g/100 mL, in water containing NaOH, HCl, dil 1:20, pH 9.0) were irradiated in quartz cuvettes, by means of Philips HPW125 or Philips PL-S 9W/12 lamps, mainly emitting at 365 or 312 nm, respectively. Light doses were measured with a Model 97503 radiometer (Cole Palmer, Niles, IL, USA) equipped with CX-365 or CX-312 sensors. The photolysis in water and methanol solution was followed by means of a

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Cary 50 UV−vis spectrophotometer and then analyzed by HPLC (Perkin-Elmer Series 200 instrument) equipped with a diode array detector (LC-235) set at 220 and 260 nm and a Gemini C18 column (5 μm, 250 × 4 mm, Phenomenex, Bologna, I), eluted with 0.5% MeOH in water containing 0.05% trifluoroacetic acid (TFA) linearly increased up to 20% MeOH within 25 min, at a flow rate of 1 mL/min. Quantitative evaluation was made on three distinct experiments. The irradiated solutions were then concentrated under reduced pressure and submitted to TLC (silica gel plates, 0.2-mm thickness, E. Merck, Darmstadt, D), eluted with ethyl acetate/methanol/formic acid, 90:10:0.05 or 95:5:0.05 v/v/v, for samples irradiated in water or methanol, respectively. For the visualization of the bands corresponding to the photoproducts, the plates were heated on a heater plate at 80 °C for 5 min and then visualized under 254 nm lamp illumination. Samples of 5-FU were also irradiated in the solid state and in a commercial cream (Efudix dermatological cream from Carlo Erba, Milano, Italy), both as 0.5-mm layers between two optical glasses whose transmittance was higher than 95%. For the HPLC analysis, the solid was dissolved in methanol and the cream in a tetrahydrofuran−water mixture (9:1, v/v). In these cases, HPLC elution was performed isocratically (water−acetonitrile 52:48, v/v containing 0.05% trifluoroacetic acid).

Mass Spectrometric Measurements. These analyses were carried out on a API-TOF Mariner spectrometer (PerSeptive Biosystems, Stratford, TX, USA), and the injection of the samples was achieved with a micrometric syringe pump (Harvard Apparatus, Holliston, MA, USA). The MS experiments were performed in the positive or negative-ion mode as necessary for improving sensitivity. Full-scan mass spectra were recorded between 150 and 2500 mass units with a scan rate of 4 s per scan in MS mode. The source temperature was 25 °C, and the desolvation temperature was 140 °C. The ESI probe voltage was 4.0 kV. The ESI drying and nebulizing gas was nitrogen. Nozzle potential was 90 V. Samples were dissolved in methanol containing 0.5% formic acid or ammonium hydroxide and infused at a flow rate of 10 μL/min. Data were acquired by a Mariner Workstation 4.0 and processed by Data Explorer 4.0 (PerSeptive Biosystems, Framingham, MA, USA). Calibration was achieved with a mixture of histidine ($[M - H]^-$ m/z 154.0622) and 4,6-dimethyl-7-hydroxycoumarin ($[M - H]^-$, m/z 189.0557) and 4,6-dimethyl-7-hydroxycoumarin ($[M + H]^+$, m/z 189.05464) and dansylglycyltryptophan ($[M + H]^+$ m/z 495.169667) in the negative and positive-ion mode, respectively.

NMR Measurements. Standard ^{1}H and ^{13}C spectra and homocorrelated (COSY) and heterocorrelated (HMBC) experiments were carried out with a Bruker AMX300 spectrometer (300 MHz for ^{1}H, 75 MHz for ^{13}C). Samples were dissolved in deuterated Me_2SO; J values are given in Hz.

Cytotoxic Activity in Sensitive Tumor Cells before and after UVB Irradiation. HCT-15 (human colorectal adenocarcinoma) and HeLa cells (human epitheloid cervical carcinoma) were obtained from ATCC (Rockville, MD). Cell lines were maintained in the logarithmic phase at 37 °C in a 5% carbon dioxide atmosphere, using RPMI-1640 medium for HCT-15 cells (Euroclone, Celbio, Milan, Italy) and F-12 HAM'S (Sigma Aldrich, Milano, Italy) for HeLa cells, containing 10% fetal calf serum (Biochrom-Seromed GmbH&Co, Berlin, Germany) and supplemented with 25 mM HEPES buffer, L-glutamine, and the antibiotics penicillin (50 units/mL) and streptomycin (50 g/mL). The growth inhibitory effect toward tumor cell lines of non-irradiated and irradiated drugs was evaluated by means of MTT (tetrazolium salt reduction) assay.[8] Briefly, 5 × 10^3 cells/well were seeded in 96-well microplates (Cellstar, Greiner Bio-One GmbH, Frickenhausen, Germany) in growth medium (100 μL) and then incubated at 37 °C in a 5% carbon dioxide atmosphere. After 24 h, the medium was removed and replaced with a fresh one containing different concentrations (6.25 to 200 μM) of both intact and photolyzed 5-FU (exposed to 30 J/cm^2 UVB irradiation in water, which induced almost total photolysis). Triplicate cultures were established for each treatment. After 72 h, each well was treated with 10 μL of a 5 mg/mL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) saline solution (Sigma Aldrich), and after 5 h of incubation, 100 μL of a sodium dodecylsulfate (SDS) solution in 0.01 M HCl was added. Following overnight incubation, the inhibition of cell growth induced by the tested compounds was detected by measuring the absorbance of each well at 570 nm using a Bio-Rad 680 ELISA microplate reader. Mean absorbance for each drug dose was expressed as a percentage of the control untreated well absorbance and plotted versus drug concentration. IC_{50} values represent the drug concentrations that reduced the mean absorbance at 570 nm to 50% of those in the untreated control wells.

Cytotoxic Activity in NCTC-2544 Keratinocytes, NIH-3T3 Fibroblasts, and MCF-7 Breast Cancer Cells before and after UVB Irradiation. Human NCTC-2544 keratinocytes and MCF-7 breast cancer cells resistant to 5-FU were grown in Dulbecco's modified Eagle's medium (Sigma Aldrich) supplemented with 115 U/mL of penicillin G (Invitrogen, San Giuliano Milanese, Milano, Italy), 115 μg/mL streptomycin (Invitrogen), and 10% fetal bovine serum (Invitrogen). Individual wells of a 96-well tissue culture microtiter plate were inoculated with 100 μL of complete medium containing 5 × 10^3 cells. Plates were incubated at 37 °C in a humidified atmosphere of 5% CO_2 for 24 h. After the removal of the medium, cells were treated with 100 μL of 5-FU dissolved in Hank's balanced salt solution (HBSS; pH 7.2) at different micromolar drug concentrations and incubated for 1 h for NCTC-2544 and NIH-3T3 or for 24 for MCF-7. Then the plates were irradiated with increasing UVB doses (0.2, 0.4, 0.6 J/cm^2) using one or two PL-S 9 W/12 Philips lamps (280−370 nm; peak at 315 nm). To limit the incident radiation to the 310−370 nm range, a glass filter (Schott SWG-320) was used. After light treatment, drug solutions were replaced by the medium and the plates placed in the incubator for 72 h. Triplicate cultures were established for each treatment. Cell viability was assayed by the MTT test as described above. Percent of viability was obtained in triplicate experiments.

Reactive Oxygen Species Determination. As described,[9] samples containing the compounds under examination (2.2 × 10^{-5} M), p-nitrosodimethylaniline (4 × 10^{-5} M) and imidazole (4 × 10^{-5} M) in phosphate buffer (0.02 M, pH 7.3) were irradiated with increasing UVB doses (0−20 J/cm^{-2}), and their absorbance at 440 nm was then measured for singlet oxygen production. Superoxide anion was determined following Pathak and Joshi:[10] samples containing the compounds under examination (10^{-5} M) and nitroblue tetrazolium (1.6 × 10^{-4} M) in carbonate buffer (pH 10) were irradiated with increasing UVB doses, and their absorbance at 560 nm was measured.

Carbonylated Proteins. Bovine serum albumin (BSA, 2 mg/mL in PBS) was chosen as a model protein for this assay. The protein carbonyl groups were determined spectrophotometrically following the procedure of Reznick.[11] Five hundred-microliter BSA samples were incubated at room temperature for 30 min with the compounds (10^{-4} M final concentration) and irradiated with increasing doses of UVB (0−15 J/cm^2). Five hundred microliters of the derivatization reagent (10 mM 2,4-dinitrophenylhydrazine (DNPH), 2.5 M HCl) was added, and samples were allowed to stand in the dark at room temperature for 1 h with vortexing every 10 min; then samples were precipitated with 20% trichloroacetic acid (TCA) and centrifuged for 5 min. The supernatants were discarded, and the protein pellets were washed three times with 1.5-mL portions of ethanol/ethyl acetate (1:1, v/v) to remove any free reagent. Samples were then resuspended in 6 M guanidine hydrochloride (dissolved in 20 mM phosphate buffer, pH 2.3) and allowed to stand at 37 °C for 15 min with vortex mixing. Carbonyl contents were determined from reading the absorbance at 375 nm using a molar absorption coefficient (ε) of 22,000 $M^{-1} \cdot cm^{-1}$. DNPH-treated and nontreated BSA samples (dark and irradiated) were used as controls.

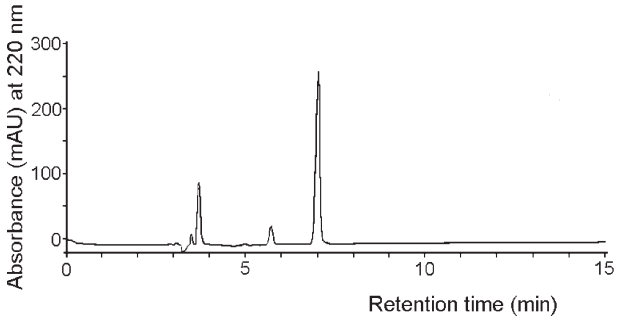


Figure 1. HPLC analysis of a UVB irradiated (20 J/cm^2) aqueous solution of 5-FU (1×10^{-5} M).

Photosensitization of Amino Acids. Solutions of 5-FU (1×10^{-3} M) were irradiated in quartz cuvettes (10 mm) in the presence of 10^{-3}–10^{-4} M amino acids (cysteine, serine, and methionine, obtained from Sigma Aldrich) in water/ethanol solution (1:1). To monitor the photomodification of amino acids, MS analyses were performed as already described.

RESULTS

5-FU Photolysis in Water. The sensitivity of 5-FU to UVA and UVB light was studied by means of spectrophotometric analysis, followed by TLC and HPLC separation of the photoproducts formed.

The evolution of 5-FU with increasing irradiation UVA and UVB doses (up to 20 J/cm^2) was followed by UV/vis absorption spectroscopy in water (1×10^{-5} M solutions). The UV absorption spectrum of 5-FU shows a maximum at around 270 nm with a small shoulder between 300 and 320 nm. By increasing UVB irradiation (up to 20 J/cm^2), a strong decrease in spectral intensity was detected, without the appearance of new spectral features. Conversely, the irradiation of the same solution with UVA light did not induce significant modifications in the UV absorption spectrum, even under high light doses (up to 30 J/cm^2). For this reason, the following experiments were performed using only UVB light.

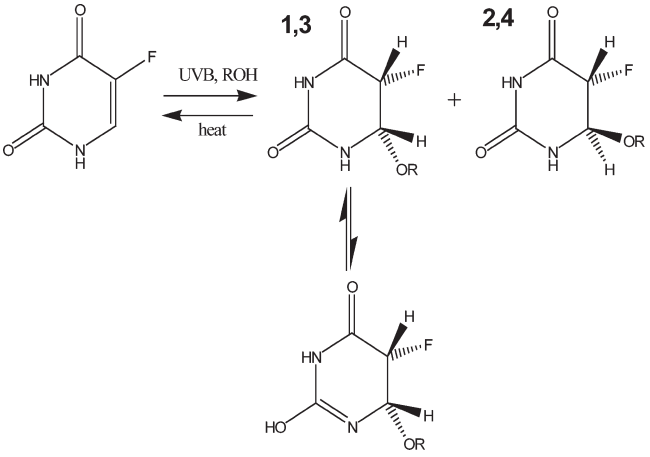
Aqueous UVB irradiated solutions (1×10^{-4} M) were also analyzed by HPLC, through which the yield of photodegradation was evaluated. The chromatogram (Figure 1) shows the presence of unmodified 5-FU (t_R = 7.0 min) along with two peaks at 3.4 and 5.7 min. By comparison with authentic compounds (see later), they were identified as the two photoproducts formed following the photoaddition of water to the 5,6 double bond of 5-FU.

HPLC experiments also allowed the rate of 5-FU photolysis to be determined. When a 1×10^{-5} M solution was exposed to increasing UVB doses (5, 15, and 30 J/cm^2), photolysis accounted for about 30 ± 2.5, 65 ± 3.1, and >95%, respectively

Characterization of the Photoproducts. For preparative purposes, larger volumes of 10^{-3} M solutions in water were irradiated with 30 J/cm^2, the solvent removed under reduced pressure, and the mixture resolved by TLC using ethyl acetate/methanol/formic acid, 90:10:0.05 v/v/v as the eluting mixture. This procedure allowed photoproducts to be isolated in sufficient amounts for structure elucidation.

As the photoproducts do not absorb around 250 nm, they could not be visualized on the TLC plate by means of mineral light, only 5-FU, which is visible in these conditions, was visualized. In order to reveal the photoproducts on the TLC plate, a small slice of it was cut and heated; in this way, the photoproducts reconverted to 5-FU, and the corresponding bands became visible under 254-nm light, as for 5-fluoro-6-hydroxyhydrouracil.[1]

Scheme 1. 5-FU and Its cis (1 and 3) and trans (2 and 4) Photoadducts with Water (R = H) and Methanol (R = CH_3), Respectively[a]



[a] All photoproducts were found to be in equilibrium with their tautomeric forms.

^{1}H- and ^{13}C NMR Analysis. For comparison, the analytical data for 5-FU (see Scheme 1) relevant for the assignment of the structure of photoproducts are reported here: TLC, R_f = 0.59. HPLC, t_R = 7.0 min. ^{1}H NMR, (300 MHz, Me_2SO), δ 10−11 (very br, 2H); 7.70 ppm (d, J = 5.9 Hz, 1H). ^{13}C NMR (75 MHz, Me_2SO), δ 167.5 (d, J = 26.1 Hz), 159.6, 149.3 (d, J = 227.3 Hz), 135.9 ppm (d, J = 31.7 Hz)

Photoproduct ***1***. TLC, R_f = 0.53. HPLC, t_R = 3.4 min. ^{1}H NMR (300 MHz, Me_2SO), δ 10.5 (br); 8.89 (br, 1H)*; 8.62 (s); 5.39 (dd, J = 4.0 and 45.9 Hz, 1H); 4.93 ppm (dd, J = 3.9 and 8 Hz, 1H). *Identified as H-1 through its coupling with H-6 in the COSY spectrum. ^{13}C NMR (75 MHz, Me_2SO), δ 167.5 (d, C-4, J = 21.7 Hz); 165.9 (s, C-2α); 152.8 (s, C-2β); 84.7 (d, C-5, J = 187 Hz); 80.4 ppm (d, C-6, J = 23.5 Hz).

Photoproduct ***2***. TLC, R_f = 0.67. HPLC, t_R = 5.7 min. ^{1}H NMR (300 MHz, Me_2SO), δ 10.5 (br); 8.86 (br, 1H)*; 8.49 (s); 5.39 (dd, J = 3.0 and 46.6 Hz, 1H); 4.93 ppm (dd, J = 3.9 and 8 Hz, 1H). *Identified as H-1 through its coupling with H-6 in the COSY spectrum. ^{13}C NMR (75 MHz, Me_2SO), δ 167.5 (d, J = 21.7 Hz); 165.9 (s, C-2α)*; 152.8 (s, C-2β)*; 84.7 (d, J = 187 Hz); 80.4 ppm (d, J = 23.5 Hz). *α and β refer to the two tautomers in Scheme 1.

The two photoproducts originate therefore from the addition of a water molecule to the 5,6 double bond of 5-FU leading to 5,6-dihydro-5-fluoro-6-hydroxyuracil, as already described for UVC irradiation.[2] Moreover, both TLC and HPLC show that two distinct photoproducts form, having strictly similar spectral features. The addition of the hydroxyl group may occur on either side of the plane of the molecule, thus accounting for the formation of two isomers in which the fluorine and the oxygen atoms are in the cis or trans positions. As the coupling constant between the 5-H and 6-H is higher and that between 5-F and 6-H is lower in photoproduct **1** than in **2**, a cis structure is proposed for the former and a trans for the latter.

The ^{13}C NMR spectrum of both **1** and **2** reveals the presence of the signals of C-4, 5, and 6 as doublets due to their coupling

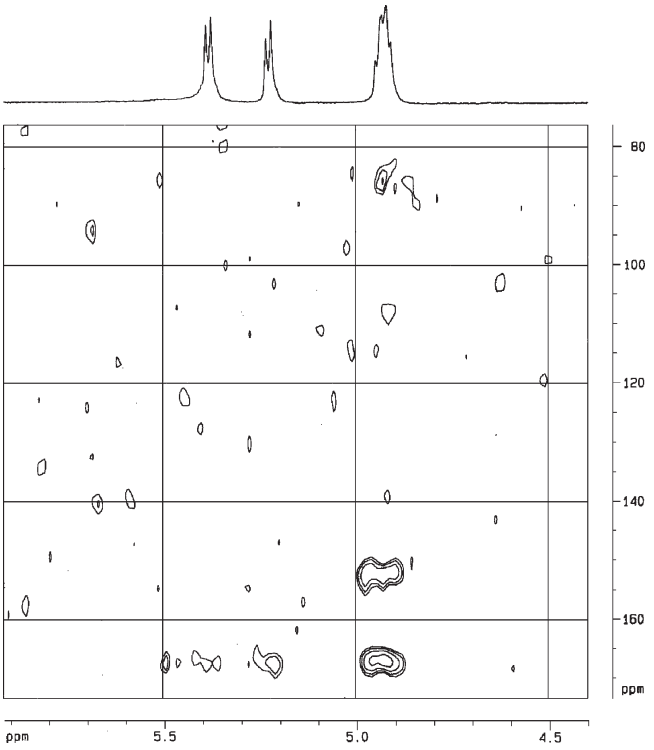


Figure 2. Relevant section of the HMBC spectrum of photoproduct **1**.

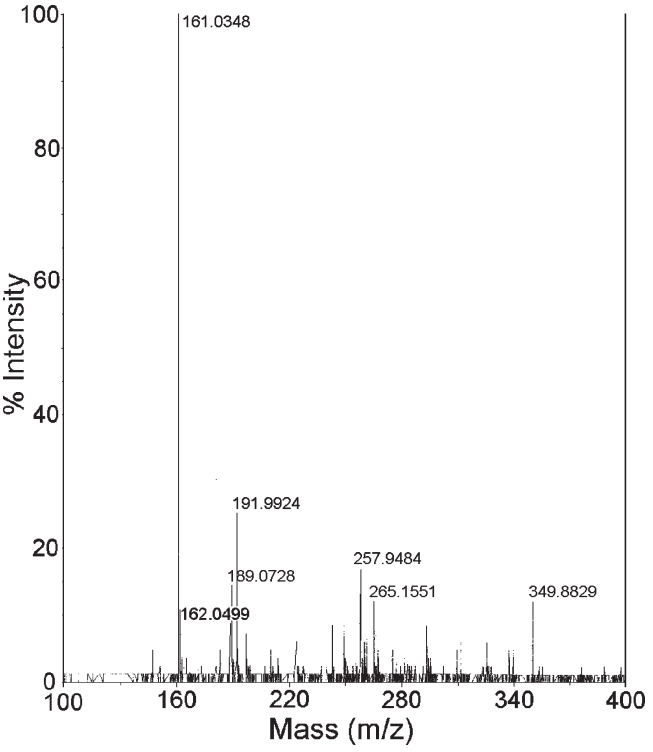


Figure 3. ESI mass spectrum (negative ion mode) of a methanol solution of 5-FU (10^{-4} M) irradiated with UVB light (20 J/cm^2).

with fluorine and of C-1 as a singlet. A further signal appears at 152.2, however, suggesting a possible tautomerism between positions 3 and 4 (Scheme 1). This hypothesis is supported by the HMBC spectrum of **1** (Figure 2), which shows interactions of H-5 with a carbonyl, reasonably C-4, and of H-6 with one or both carbonyls, and C-5.

Irradiation in Methanol. 5-FU was also irradiated in methanol. The corresponding HPLC profile (not shown) revealed that four photoproducts form, two of which are also found in water due to the water content of commercial methanol. TLC analysis (ethyl acetate/methanol/formic acid, 95:5:0.05 v/v/v) of the mixture afforded two compounds which were characterized as follows.

Photoproduct ***3***. TLC, R_f = 0.62. HPLC, t_R = 6.4 min. ^{1}H NMR (300 MHz, Me_2SO), δ 10.8 (br); 8.85 (br, 1H)*; 8.52 (s); 4.84 (dd, J = 4.0 and 45.9 Hz, 1H); 4.72 (m, 1H); 3.33 ppm (s, 3H). *Identified as H-1 through its coupling with H-6 in the COSY spectrum. ^{13}C NMR (75 MHz, Me_2SO), δ 167.0 (d, C-4, J = 21.6 Hz); 166.4 (s, C-2α,); 152.38 (s, C-2β); 84.3 (d, C-5, J = 190.7 Hz); 79.9 (d, C-6, J = 22.4 Hz); 55.1 ppm (OCH_3).

Photoproduct ***4***. TLC, R_f = 0.77. HPLC, t_R = 10.8 min. ^{1}H NMR (300 MHz, Me_2SO), δ 10.5 (br); 8.85 (br, 1H)*; 8.52 (s); 5.46 (dd, J = 3.2 and 46.7 Hz, 1H); 4.72 (m, 1H); 3.30 ppm (s, 3H). *Identified as H-1 through its coupling with H-6 in the COSY spectrum. ^{13}C NMR (75 MHz, Me_2SO), δ 166.3 (s, C-2α)*; 165.5 (d, J = 18.8 Hz); 151.7 (s, C-2β)*; 83.0 (d, J = 177.2 Hz); 80.4 (d, J = 20.7 Hz); 55.3 ppm (s, OCH_3). *α and β refer to the two tautomers in Scheme 1.

The crude irradiation mixture was also examined by mass spectrometry with the electrospray technique (Figure 3). The negative-ion analysis clearly shows the presence of a main species having m/z 161.035, indicating the presence of the photoaddition product between 5-FU and methanol.

Stability and Photostability Studies in the Solid State and in Drug Formulations. The HPLC method developed for the photolysis studies in solution was also applied to the analysis of 5-FU irradiated in the solid state or in two final topical or systemic drug formulations.

In the Solid State. UVB irradiation (0, 5, 15, 30 J/cm^2) of a 0.5-mm layer of 5-FU in powder did not show any change in HPLC chromatograms. Therefore, 5-FU proved stable and photostable when stored in the solid state.

In a Systemic Pharmaceutical Formulation for Injection. A 5% (3.8×10^{-1} M) 5-FU aqueous solution for injection (Teva) was irradiated either in its glass vial (immediate pack) or in a quartz cuvette under increasing UVB doses (5, 15, and 30 J/cm^2). In both containers, photolysis of 5-FU was negligible. As one problem indicated by some patients concerns the block of the infusion device, the same sample was tested for stability under different temperatures (from 4 to 37 °C) or daily light exposure in its immediate pack (glass vial) and in the reservoir. Drug stability was checked every two days by means of HPLC.

The samples showed total stability in all the conditions tested until 30 days, except for the sample stored at 4 °C in which, after one week, a visible precipitation occurred, which resulted in the loss of 6% of the active principle concentration. As a consequence, not only the right dosage can be impaired but the precipitate can interfere with drug administration through the portable infusion pump during the treatment of the patient.[12]

In a Topical Pharmaceutical Formulation. The photolysis of 5-FU was studied in a commercial formulation for topical use (Efudix cream, containing 5% of 5-FU): UVB irradiation (5, 15, and 30 J/cm^2) of a 0.5-mm layer of the cream resulted in 5, 20, and 37% degradation of the active principle, respectively. However, the decrease of the peak of 5-FU was not accompanied by

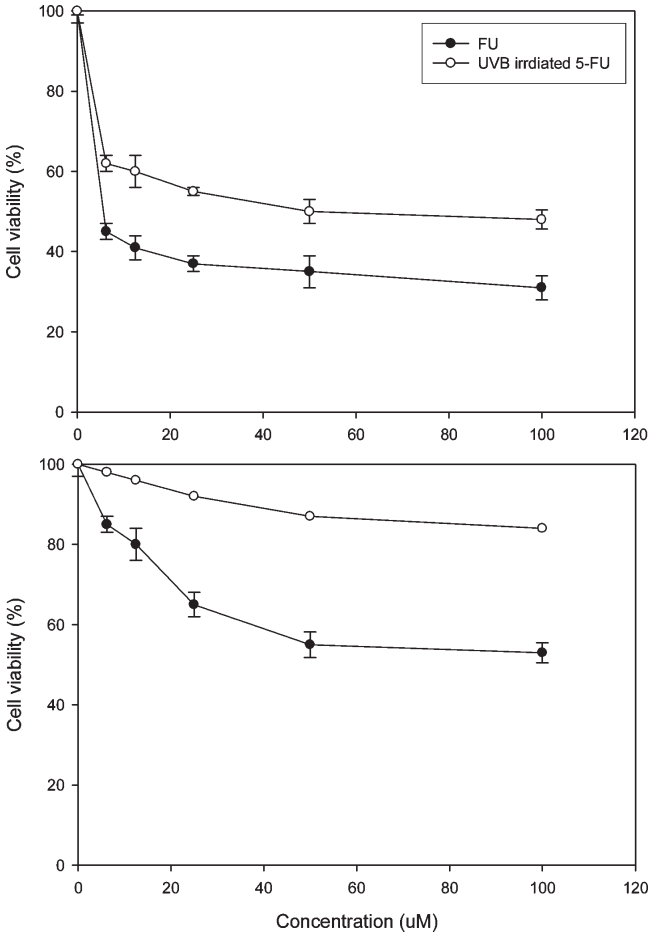


Figure 4. Dose−response curves of HeLa (upper) and HCT-15 (lower) cells incubated for 72 h with unmodified and photomodified 5-FU. Cytotoxicity was determined by means of the MTT test.

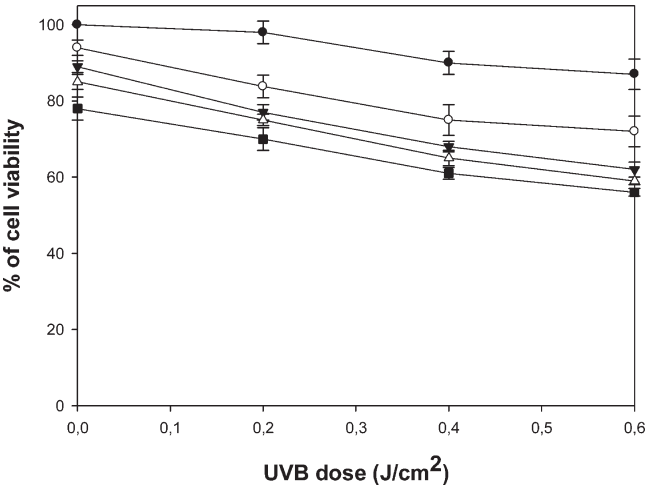


Figure 5. NCTC-2544 cell viability (%) after 1 h of incubation with 12.5 (○), 25 (▼), 50 (Δ), 100 μM (■) 5-FU followed by irradiation with increasing doses of UVB light. Cells irradiated without 5-FU served as the control (●).

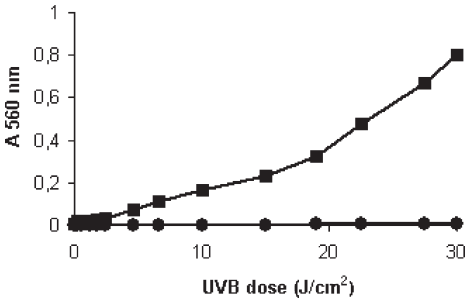


Figure 6. Superoxide anion formation induced by 5-FU under UVB irradiation (■), following the Pathak and Joshi procedure.[10] A sample irradiated without the drug served as the control (●).

the appearance of new significant peaks in the chromatograms, probably because the variety of excipients present in the cream and possessing hydroxyl groups (parabens, glycerol, propylene glycol, stearylic alcohol) are able to react through nucleophilic photoaddition to the 5,6 double bond as in solution.

Cytotoxic Activity before and after UVB Photomodification in Sensitive Cancer Cells. Intact and photomodified (>80%, after 30 J/cm^2 of UVB) 5-FU samples were examined for their cytotoxic properties on human squamous cervical adenocarcinoma (HeLa) and colon adenocarcinoma (HCT-15) cell lines which proved sensitive to 5-FU among a panel of cell lines tested (not shown). Cell viability was measured by means of the MTT test (Figure 4). The figure shows the dose-survival curves with increasing concentrations of intact and photomodified 5-FU after 72 h of incubation of HeLa (panel A) and (panel B) cells.

In both cell lines, intact 5-FU showed antiproliferative efficacy much greater than that after photolysis; in particular, against HCT-15 cells 5-FU had an average IC_{50} value that was at least 25 times lower than that obtained with photoirradiated 5-FU.

Phototoxicity in Fibroblasts and Keratinocytes. The phototoxic activity of 5-FU was studied in vitro in two model cell lines, NCTC-2544 keratinocytes and NIH-3T3 fibroblasts). A very similar trend was seen with both cell lines: after 1 h of incubation, 5-FU was cytotoxic (loss of viability of about 10 to 20%) when the cells were incubated in the dark at concentrations varying from 12.5 to 100 μM; cytotoxicity was slightly higher (loss of viability from 20 to 40%) when they were further irradiated with UVB light (0.2, 0.4, and 0.6 J/cm^2) at all the concentrations used (see Figure 5 for keratinocytes). When incubation with the drug was prolonged to 24 h before irradiation, both cyto- and phototoxicity were not much higher (30% to 40%). In all experiments, the difference in viability among the various concentrations used was small, 100 μM being a bit more toxic than 12.5 μM. If compared with the effect of UVB irradiation alone on the cells, 5-FU induced about 20% more cell death with respect to cells irradiated without drug treatment. The same experiments with UVA light did not have any effect on cell viability. Therefore, from our results 5-FU can not be considered a strong photosensitizer, although photosensitizing reactions are reported as side effects in some patients receiving topical or systemic treatment (ref 4 and references therein).

Determination of Reactive Oxygen Species. Production of reactive oxygen species is one of the mechanisms underlying drug induced photosensitization damage. 5-FU generated the superoxide anion ($O_2^{\bullet -}$) efficiently under UVB irradiation (Figure 6), while singlet oxygen ($^1O_2$) production was very low (data not shown). Especially at the highest doses of UVB used for this experiment (15 and 20 J/cm^2), the formation of superoxide anion is comparable to that of other known photosensitizers[13,14]

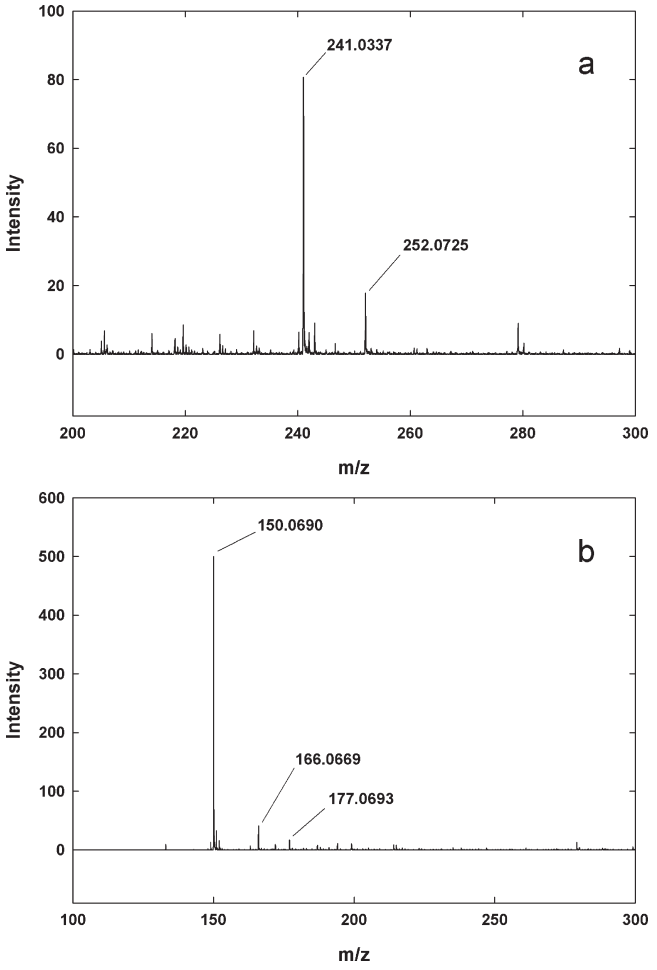


Figure 7. ESI-mass spectrum (positive ion mode) of a sample of cysteine (a) and of methionine (b) irradiated with 5-FU.

and is most likely responsible for the protein and amino acid photooxidation shown below.

Protein Photooxidation: Determination of Carbonyl Content. Photooxidation is a kind of protein damage that could be responsible for phototoxic effects of the drugs. The most common products of protein oxidation are the protein carbonyl derivatives of amino acids, mainly of lysine, arginine, proline, and hystidine.[15] Therefore, carbonylated BSA (chosen as the model protein) was taken as a measure of oxidative protein damage induced by 5-FU under UVB irradiation; the amount of carbonyls formed was calculated on the basis of the increase of absorbance at 375 due to the reaction with DNPH followed by the formation of the corresponding hydrazones (ε_{375} = 22,000 $M^{-1}\cdot cm^{-1}$). Photooxidation of BSA alone, before and after irradiation, was measured as the blank reference. 5-FU demonstrated the formation of high levels of carbonyls in BSA under increasing doses of UVB light (5.3 ± 1.8, 10 ± 2.4, and 9.8 ± 2.1 nmol/mg BSA at 5, 10, and 20 J/cm^2, respectively). Protein photooxidation can therefore play a significant role in photosensitization effects induced by 5-FU in patients.

Photomodification of Amino Acids Detected by MS. If nucleophilic solvents are able to attack the 5,6 double bond, the same can occur with nucleophilic groups of proteins, such as the −OH, −SH, and $-NH_2$ groups. For this reason, we studied the photoaddition of some amino acids to 5-FU. 5-FU was irradiated in the presence of cysteine, serine, and methionine in an aqueous/ethanol solution (1:1). As these experiments were carried out in water or water−alcohol mixture, the solvent was by far the strongest nucleophile. As a consequence, these photoreactions gave rise mainly to photoproducts with the solvent and only to trace amounts of photoaddition or photooxidation products with amino acids, insufficient for further NMR characterization. Therefore, only MS analysis of the irradiated solutions was performed. Figure 7a shows the ESI mass spectrum in the positive ion mode of a sample of cysteine (MW = 121) incubated with the drug (MW = 130) and irradiated with UVB. Along with the peak of cystine (m/z 241.034), generated from oxidation induced by 5-FU plus UVB light, the peak of a monoadduct of 5-FU with the amino acid (m/z 252.072) is present. Both peaks were absent in the sample kept in the dark (not shown), thus demonstrating that irradiation was able to induce drug−amino acid photoaddition and also oxidative damage toward the substrate. The same photoaddition was seen with serine (m/z 234.053; ESI mass spectrum in the negative ion mode, not shown). On the contrary, with methionine (m/z 150.069) no photoadduct was found (Figure 7b; ESI mass spectrum in the positive ion mode). Indeed, with this amino acid, having the easily oxidizable methylthio group, only an oxidation product formed (monooxidized, m/z 166.067). The absence of a photoadduct with methionine also suggests that covalent photoaddition of 5-FU with amino acids, such as serine and cysteine, involves only the side chain and not the primary amino group, as shown for fluphenazine.[16] It is also interesting to note that the peak at m/z 177.069 (Figure 7b) corresponds to the photoaddition of the solvent (ethanol) to 5-FU.

It must be pointed out that the low yield of these photoreactions prevents isolation of the covalent adducts of the amino acids with 5-FU for spectroscopic characterization; therefore, the exact structure of these photoaddition products is still not definite.

However, covalent binding of 5-FU to amino acids suggests a further photomodification of proteins. This damage could lead to the formation of photoantigens and trigger the photoallergic reactions found in vivo.

Antiproliferative Activity of 5-FU in Combination with UVB Light on MCF-7 Resistant Cells. 5-FU shows antiproliferative activity only on selected cell lines as many cells are resistant to its cytotoxic effect. Since some cases of higher efficacy of 5-FU under occasional exposure of the patients to solar radiation are reported and 5-FU can also be used as a radiosentitizer,[5] we studied the effect of 5-FU on MCF-7 resistant cells in combination with UVB light, in order to demonstrate whether the cytotoxic effect of 5-FU can be enhanced by UVB irradiation.

Irradiation of cells incubated for 24 h with 5-FU induces a small decrease of viability (about 10−15%) with respect to the samples kept in the dark, independently of the drug concentration applied. Therefore, the higher effect found under irradiation seems to arise from an additive inhibitory effect on cell growth of both drug and light, other than a typical photosensitizing effect, as already suggested by Hamaoka.[17]

DISCUSSION

In this article, the effect of UVB light on 5-FU was studied in order to define whether UVB light is able to (i) modify the molecule, thereby altering its cytotoxic activity in sensitive cells (HeLa e HCT-15), (ii) trigger photosensitizing effects on

NCTC-2544 cells, or (iii) improve the antiproliferative activity of 5-FU in resistant MCF-7 cells. Our attention was focused on UVB light since UVA did not induce significant changes to the molecule and did not modify its reactivity toward biological substrates.

Martindale suggests the protection of 5-FU from light, but no studies are present in the literature about its UVB/UVA light degradation in aqueous solutions and in drug formulations. We have demonstrated that in diluted water and methanol solutions 5-FU is unstable under UVB irradiation forming photoproducts derived from the photoaddition of the solvent to the 5,6 double bond. It also proved photolabile in a commercial formulation for topical use, thus confirming the requirements of the pharmacopeias for light protection of this drug. Instead, the drug proved stable to UVB in the solid state and in a systemic concentrated formulation. Our study has also demonstrated the high stability of 5-FU in a disposable infusion pump under different temperature and light conditions up to 1 month, except for the sample stored in the refrigerator. Photolysis of 5-FU caused a loss of its antiproliferative activity in HCT-15 and HeLa tumor cell lines sensitive to 5-FU. It is reasonable to think that the same problem can occur in systemically treated patients, thus losing therapeutic activity;[18] indeed, 5-FU reaches 10^{-5}–10^{-6} M concentration in the steady state during Teva infusion,[19] this concentration being very sensitive to photodegradation.

In vitro phototoxicity induced by 5-FU was detected in NCTC-2544 and 3T3 cell lines which was slight compared to that of other known photosensitizers. A little improvement in the antiproliferative activity of 5-FU on MCF-7 resistant cells in combination with UVB irradiation was also evidenced. However, the UVB dose that can be used with cells is quite low (not higher than 0.6 J/cm^2). When higher UVB doses were allowed in other experiments with isolated substrates, 5-FU demonstrated efficient damage of the biological substrates employed. Indeed, we found the photoaddition of amino acids (i.e., cysteine and serine) to 5-FU as well as photooxidation of cysteine, methionine, and BSA, and high production of reactive oxygen species, in particular superoxide anion. Protein damage possibly explains the occurrence of photoallergic effects and other undesired light-induced side effects of this drug.

The same experiments made under UVA irradiation did not give significant results. Therefore, we suggest that UVB light is able to raise both 5-FU photosensitization effects and photodegradation.

In conclusion, as UVB is a component of daylight, the lack of photoprotection for the drug product during storage and administration may lead to decreased efficacy of this photolabile drug. Moreover, to prevent photosensitizing side effects, patients under 5-FU treatment should avoid sun or artificial lamp exposure particularly when 5-FU is administered topically for skin and eye diseases.

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ABBREVIATIONS

5-FU, 5-fluorouracil; DNPH, 2,4-dinitrophenylhydrazine; COSY, H−H correlation spectroscopy; HMBC, heteronuclear multiple bond correlation; UVB, ultraviolet light, class B (290−315 nm).

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