



Lack of in vitro protection by a common sunscreen ingredient on UVA-induced cytotoxicity in keratinocytes

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Abstract

As an extension of our previous investigations on sunscreen ingredients, the present work was aimed at assessing the possible protective effects of a common UVA-absorbing agent, Parsol 1789 (4-*tert*-butyl-4'-methoxydibenzoylmethane) in contact with human keratinocytes under UVA illumination. Cell viability was evaluated by determining lactate dehydrogenase (LDH) release, uptake of propidium iodide and fluorescein diacetate, total protein content and percentage of cell detachment. Apoptosis was detected by recognition of translocated phosphatidylserine using annexin V-FITC uptake. Oxidative stress was evaluated through the carboxy-H₂DCFDA assay while the total oxyradical scavenging capacity (TOSC) assay was used for determining the total antioxidant capacity level in these cells. Lipid peroxidation was also assessed by checking hydroperoxide (HP) levels. The results obtained show that UVA exposure induces significant cell mortality, decrease in protein concentration, release of LDH, increase in apoptosis, oxidative stress and lipid peroxidation with a concomitant reduction in the response of the antioxidant cellular defense system. The presence of 10 μM Parsol 1789 did not minimize these UVA-induced effects, on the contrary, for some parameters measured such as lipid hydroperoxides, there was a significant enhancement. Furthermore, the presence of glutathione (GSH) alone decreased the level of ROS and lipid hydroperoxides, but in combination with Parsol 1789, this protective effect was reduced. The overall results indicate that the compound does not protect these cells from UVA exposure under our experimental conditions confirming previous findings on the lack of photoprotective efficiency of this sunscreen in contact with biologically relevant molecules. However, the biological role and significance of these results to the consequences of sunscreen use in humans are not known, hence extrapolation from laboratory experiments must be done with caution.

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1. Introduction

Changes in lifestyle and the development of leisure activities and holiday habits, as well as tanning for cosmetic purposes either by sunbathing or by using artificial tanning devices has led to a general increase in daily exposure of the skin to ultraviolet (UV) light. Depletion of the stratospheric ozone layer which protects the earth's surface from the most damaging solar UV radiations may also contribute to this increased exposure (Abarca and Casiccia, 2002). As a consequence, the hazards associated with exposure to the UVA and UVB components of sunlight, which include erythema, sunburn, photodamage (photoaging), photocarcinogenesis, damage to the eyes, alteration of the immune system of the skin and chemical hypersensitivity, are an important issue (Drobetsky et al., 1995; Taylor et al., 1990; Lucas and Ponsonby, 2002; Clydesdale et al., 2001; Armstrong and Kricger, 2001; Gruijl, 2000). The primary method of mitigating these harmful effects, besides wearing protective clothing or staying out of the sun and not using tanning devices, is the use of sunscreens (Thompson et al., 1999; Diffey et al., 2000; Gasparro et al., 1998).

Of the two types of topical sunscreens: (1) chemical sunscreens that provide protection by absorbing UV radiation; and (2) physical sunscreens that block UV radiation from reaching the skin, we have recently become interested in the chemical sunscreen, Parsol 1789 (4-*tert*-butyl-4'-methoxydibenzoylmethane). This is a widely used UVA-absorbing agent present in sunscreens and increasingly in cosmetics, to provide protection against skin damage and premature aging of the skin. It is important to mention here that the main portion of UVA radiation which contributes to about 95% of natural sunlight is not trapped by standard glass: automobile windows, verandas, conservatories, and windows in general fail to protect against UVA in contrast to UVB because the short wavelength cutoff of glass is about 320 nm. Hence, cosmetic industries now incorporate ultraviolet filters into topical products intended for daily use in the belief that year-round sun exposure needs to be controlled. Now, although Parsol 1789 is designed to absorb UVA energy, and it does so very efficiently, it has a major drawback which is its lack of photostability (Deflandre and Lang, 1988; Roscher et al., 1994; Sayre and Dowdy, 1999). In fact, its photodecomposition not only reduces its

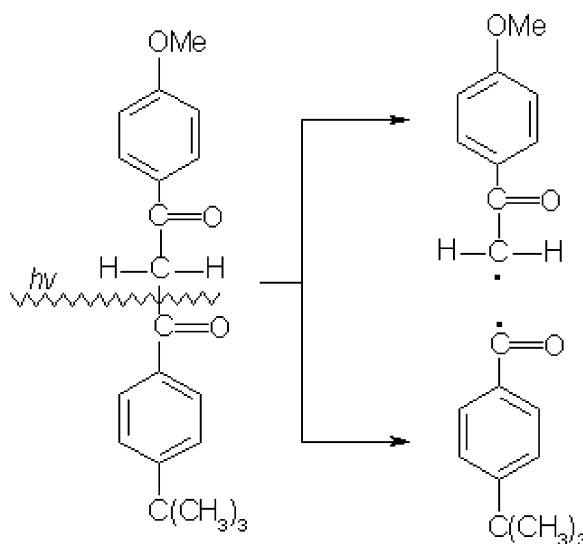


Fig. 1. Parsol 1789 (4-*tert*-butyl-4'-methoxydibenzoylmethane).

photoprotective power but it also produces an array of breakdown products deriving from the initial formation of free radicals (Fig. 1) (Schwack and Rudolf, 1995). These radicals are the most probable cause of DNA strand breaks in plasmid DNA (Damiani et al., 1999) and of oxidative modifications in bovine serum albumin (BSA) (Damiani et al., 2000), previously observed by us when both systems were illuminated in vitro in the presence of Parsol 1789. These former results have therefore prompted us to examine further the effects of this compound on cellular systems. For this purpose, a human keratinocyte cell line (NCTC2544) was chosen since keratinocytes are the relevant cell-type for most non-melanoma skin cancers, and the possible protective effect by Parsol 1789 on these cells under UVA illumination were examined.

2. Materials and methods

2.1. Materials

Parsol 1789 was obtained in the form of Eusolex 9020 from Merck (Darmstadt, Germany) and its identity was confirmed by NMR. Reagents for cell cultures were obtained from Bio-Whittaker (Walkersville, USA) while all other reagents for

molecular and biochemical analysis were purchased from Sigma Chemical Co. (St. Louis, USA). The apoptosis detection kit (Annexin V-FITC) was purchased from Alexis (Vinci-Biochem, Italy). 5-(and-6)-Carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H₂DCFDA) was purchased from Molecular Probes (Europe BV).

As UVA irradiating source, a commercial sun lamp, Philips Original Home Solarium (model HB 406/A; Philips, Groningen, Holland) equipped with a 400 W ozone-free Philips HPA lamp, UV type 3, providing an irradiance of 0.75 mW/cm² between 300 and 400 nm was used. It was always pre-run for 15 min to allow the output to stabilize. The dose of UVA was measured with a UV power pack radiometer (EIT Inc.).

2.2. Cell culture

The immortalised human keratinocyte cell line NCTC2544 (ICLC, University of Genova, Italy) used throughout this study was maintained at 37 °C and 5% CO₂/95% air in MEM (minimum essential medium) supplemented with non-essential aminoacids (1%), 2 mM L-glutamine, 100 U/ml penicillin/streptomycin and 10% (v/v) fetal bovine serum. The cells were cultured as adherent monolayers, routinely split 1:4 every 3–4 days and used for each experiment at the third passage. Forty-eight hours prior to the experiments, the cells were placed into six-well plates (diameter of each well = 3.5 cm).

2.3. Cell treatments

When monolayers of cells reached approximately 5×10^5 cells/well they were washed twice with phosphate-buffered saline (PBS). Parsol 1789 dissolved in propylene glycol and diluted at a concentration of 10 μM in PBS was added directly to the cell suspension. Control samples were treated with propylene glycol (45 mM) diluted in PBS, as well as with PBS alone.

The cells were exposed to UVA for 15 min in the presence or absence of Parsol 1789 and/or 1 mM glutathione (GSH). During UV irradiation the cell monolayer was covered with a 4 mm thick layer of PBS or PBS–Parsol solution and the multi-well plates were placed on a brass block embedded in ice at a distance of 20 cm from the light source. The incident

dose of UVA received by the cells was 207 kJ/m². After irradiation, PBS was collected and centrifuged at 1500 rpm for 10 min and the supernatant used for lactate dehydrogenase (LDH) determination. The cells were replaced in the incubator in fresh culture medium and harvested after specific incubation times. Sham-irradiated cells were similarly manipulated except that they were exposed to artificial laboratory working light.

2.4. Cell viability assays

Cell viability was assessed at 4, 24, 48 and 72 h post-treatment using fluorescein diacetate (1 μg/ml) and propidium iodide (5 μg/ml) as probes for detecting, respectively, viable and non-viable cells (Ruiz et al., 1991). Each preparation was examined on a fluorescent microscope (Nikon Eclipse E800). Cell viability was calculated on each slide, by randomly selecting 50 cells and evaluating the % of live or dead cells. For each sample, three different slides were prepared and the results reported are an average of four individual experiments.

The cell number was determined at 4 and 24 h post-treatment, by manually counting the cells after harvesting with a hemocytometer.

Protein content was determined at 4 and 24 h post-treatment using the Lowry method (Lowry et al., 1951) with bovine serum albumin as standard.

Lactate dehydrogenase activity was assayed in the supernatant media by measuring changes in absorption at 340 nm due to oxidation of NADH. The reaction mixture contained 48 mM phosphate buffer pH 7.5, 0.6 mM pyruvate, 0.18 mM NADH and supernatant medium as sample. One unit of enzyme activity corresponds to the amount of enzyme that converts 1 μmol of substrate per min; specific activity is expressed as μmol/min mg proteins (Bergmeyer and Bernt, 1974).

2.5. Apoptosis/annexin V-FITC assay

The “annexin V-FITC apoptosis detection kit” (Alexis, catalogue no. 850-020-KI02) was used for determining translocated phosphatidylserine after 24 h post-treatment. The flow cytometer used was a Coulter EPICS XL (Coulter, Miami, FL, USA). An average of 5000 cells from each sample were counted.

2.6. Sample preparation for total oxyradical scavenging capacity (TOSC) assay

After 4 and 24 h post-illumination, UVA culture medium was discarded and monolayers were washed with PBS and cells detached by brief trypsinization. Cells were suspended in PBS (1–2 mg protein/ml) and aprotinin (1 $\mu\text{g/ml}$), frozen in liquid nitrogen three times and centrifuged at 4°C in an Eppendorf microfuge at 12,000 $\times g$ for 7 min. The cell supernatant was stored at –80°C. TOSC assay values were expressed as units per milligram of protein. Protein content was determined by the Lowry method (Lowry et al., 1951).

2.7. TOSC assay

Total oxyradical scavenging capacity toward hydroxyl and peroxy radicals were determined after 4 and 24 h from treatment using the TOSC assay (Regoli and Winston, 1999). Peroxy radicals were produced by thermal homolysis of 2-2'-azo-bis-(2-methylpropionamide)-dihydrochloride (AAPH), while hydroxyl radicals were generated by an iron-ascorbate Fenton reaction. Final assay conditions were respectively for peroxy radicals and hydroxyl radical: 0.2 mM α -keto- γ -(methylthio)butyric acid (KMBA), 20 mM AAPH in 100 mM K-phosphate buffer, pH 7.4; 0.2 mM KMBA, 1.8 μM FeCl_3 , 3.6 μM EDTA, 180 μM ascorbic acid in 100 mM K-phosphate buffer, pH 7.4. The reactions were conducted at 35°C. At 12-min intervals, aliquots of 200 μl were taken from the head-space of the reaction vessels and ethylene production measured with a Hewlett-Packard (HP 4890 series) gas chromatograph equipped with a Supelco SPB-1 capillary column (30 m \times 0.32 mm \times 0.25 μm) and a flame ionization detector. For the various oxidant-generating systems, TOSC values were quantified using the equation: $\text{TOSC} = 100 - (\text{SA}/\text{CA} \times 100)$, where SA and CA are the integrated areas calculated under the least squares kinetic curve produced during the reaction course for sample (SA) and control (CA) reactions, respectively.

2.8. Carboxy- H_2DCFDA assay

After illumination, cells were incubated with 5 μM carboxy- H_2DCFDA (Molecular Probe catalog no.

Table 1
Level of hydroperoxides measured in keratinocytes before and after UVA exposure

	– UVA			+ UVA		
	– PAR	+PAR	+GSH	– PAR	+PAR	+GSH
Straight after exposure	3.17 \pm 0.8	4.02 \pm 0.9	2.96 \pm 0.4	7.29 \pm 1.9*	17.24 \pm 4.6 [#]	5.42 \pm 2.3
After 4 h from exposure	2.39 \pm 0.6	3.65 \pm 0.7	2.48 \pm 0.5	4.99 \pm 0.9*	7.89 \pm 2.1*	4.73 \pm 1.4
						12.32 \pm 3.8*
						6.47 \pm 3.1

The level of hydroperoxides ($\mu\text{mol/mg}$ protein) was measured using the FOX2 assay in keratinocytes in the presence or absence of 10 μM Parsol 1789 (PAR) and/or 1 mM glutathione (GSH), exposed or not exposed to 15 min UVA. Values represent the means \pm S.D. of three individual experiments. The statistical significance between illuminated samples and non-illuminated ones is indicated with an asterisk (* $P < 0.05$) while the significance between illuminated samples in the presence or absence of Parsol, or GSH or both, is indicated with a hash ([#] $P < 0.05$).

C-400) in fresh PBS for 30 min. Straight after illumination, the cells were trypsinized, collected, washed twice in PBS, and analysed. Fluorescence of the labelled cells was measured by using a Coulter EPICS XL flow cytometer (Coulter). Fluorescence intensity was recorded on the cells negative to propidium iodide. An average of 10,000 cells from each sample were counted.

2.9. Hydroperoxides (HPs) assay

The ferrous-oxide xylenol orange (FOX2) method was used for determining hydroperoxides. HP levels were assayed according to the principle of the rapid peroxide-mediated oxidation of Fe^{2+} to Fe^{3+} under acidic conditions (Jiang et al., 1992) slightly modified (Nourouz-Zadeh et al., 1994) using triphenylphosphine (TPP), an agent that avoids artifactual colour generation in samples which might contain substantial quantities of loosely available iron. Briefly, after exposure, cells were trypsinized, collected, centrifuged and the pellet resuspended in 1 ml PBS. The cells (0.1 mg) were then incubated at 37 °C for 30 min with and without 1 mM TPP. Then FOX2 reagent was added to each sample and incubated again at 37 °C for 30 min in a shaking water bath. After centrifugation (2000

$\times g$ for 5 min), the supernatants were monitored at 560 nm.

2.10. Statistical analysis

Two-way analysis of variance (ANOVA) was used to evaluate the statistical difference for all the data (a P -value of <0.01 was considered statistically significant) except in the case of the data reported in Table 1 where the Student's t -test was used. Data are presented as mean \pm S.D.

3. Results

The main purpose of this study was to determine the protective effects, if any, of the chemical sunscreen Parsol 1789, on a human keratinocyte cell line when exposed to UVA light. The results obtained for the control samples, i.e. with propylene glycol or with PBS were similar, therefore only results of the control samples in PBS are reported.

3.1. Cell viability

As starting point, the cell-killing property of UVA on keratinocytes and the possible role of Parsol 1789

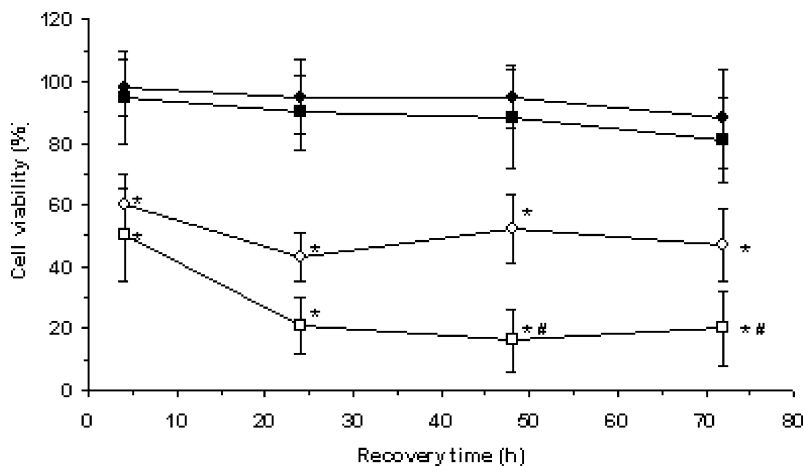


Fig. 2. Viability curves obtained from cells exposed to 15 min of UVA in the absence or presence of 10 μM Parsol 1789 at 4, 24, 48, and 72 h post-UVA illumination, using propidium iodide and fluorescein diacetate staining. (●) Non-illuminated cells without Parsol 1789; (■) non-illuminated cells treated with Parsol 1789; (○) illuminated cells without Parsol 1789; and (□) illuminated cells treated with Parsol 1789 (mean values \pm S.D. $n = 6$). The differences between illuminated samples and non-illuminated ones are always statistically significant ($*P < 0.01$) likewise between illuminated samples in the presence or absence of Parsol 1789 at 48 and 72 h ($\#P < 0.01$).

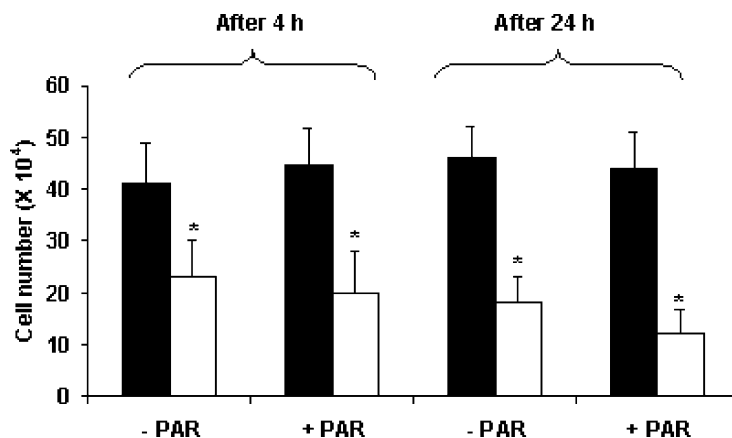


Fig. 3. Cell number in cell populations exposed to 15 min of UVA in the absence (–PAR) or presence (+PAR) of 10 μ M Parsol 1789 at 4 and 24 h post-UVA illumination. Black bars correspond to non-illuminated cells whereas white bars correspond to illuminated cells. The cell number is expressed as the number of attached cells in the culture medium (mean values \pm S.D. $n = 6$). The differences between non-illuminated and illuminated samples are always statistically significant whether in the presence or absence of Parsol 1789 ($*P < 0.01$).

in minimizing this effect was examined through four different tests.

Popular methods for quantitatively discriminating between viable (intact plasma membrane) and dead (damaged plasma membrane) cells use differential staining techniques hence, propidium iodide and fluorescein diacetate were first used to visualize dead and live cells, respectively. Viability was monitored at 4, 24, 48 and 72 h after UVA exposure, in cells

treated with and without Parsol 1789. Fig. 2 shows clearly that UVA exposure induced significant cell mortality (approximately 40%) with respect to the non-irradiated cells already after 4 h from exposure and this loss increased up to 24 h after which it remained constant. Cells irradiated in the presence of Parsol 1789 showed 50% mortality in the first 4 h post-illumination and thereafter, mortality increased steadily up to 48 h (about 70%). The concentration of

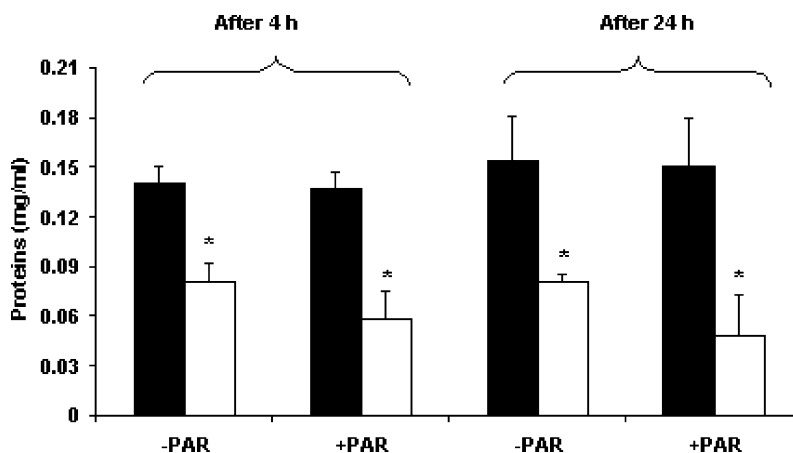


Fig. 4. Total protein concentration in cells exposed to 15 min of UVA in the absence (–PAR) or presence (+PAR) of 10 μ M Parsol 1789 at 4 and 24 h post-UVA illumination. Black bars correspond to non-illuminated cells whereas white bars correspond to illuminated cells (mean values \pm S.D. $n = 3$). The differences between non-illuminated and illuminated samples are always statistically significant whether in the presence or absence of Parsol 1789 ($*P < 0.01$).

10 μM was chosen after having performed preliminary toxicity experiments using different concentrations (1, 10, 50 and 100 μM) because higher concentrations of the compound were toxic even without exposure to UVA, while at 1 μM it had no effect.

Secondly, cell viability was determined by counting the attached cells. Counting was done after 4 and 24 h from exposure to UVA. As shown in Fig. 3, exposure to UVA induced cell mortality, which is followed by detachment of the cells from the monolayer and loss in cell number (about 45–55%) after 4 and 24 h. The samples illuminated and treated with Parsol 1789 show a similar loss in cell number after 4 h from exposure and a slightly higher loss after 24 h.

Another assay consisted in evaluating protein concentration in the cell population which is a valid method for measuring in vitro cytotoxicity (Li and Zhang, 2002). This analysis indicates that UVA exposure induces loss in proteins at 4 and 24 h after illumination and that further loss can be seen even in the cells treated with Parsol 1789 (Fig. 4).

The fourth assay was based on the release of lactate dehydrogenase due to plasma membrane alterations. Fig. 5 shows that during 15 min of exposure to UVA, the cells did not release LDH whereas after 4 and 24 h release from the cells is significantly higher in exposed cells with respect to those not exposed. The

cells illuminated and treated with Parsol 1789 also show a significant release in LDH with respect to those not illuminated.

3.2. Apoptosis

During apoptosis, the mode of suicidal cell death that occurs under normal physiological conditions, several changes take place including loss of asymmetry in phospholipids at the cell surface membrane. This alteration can be detected using annexin V, a calcium-dependent anti-phosphatidylserine antibody which recognizes the translocated phosphatidylserine. By using this staining technique in combination with propidium iodide followed by flow cytometry analysis, Fig. 6 shows that keratinocytes exposed to UVA undergo apoptosis, but that the simultaneous presence of Parsol 1789 enhances this cellular event.

3.3. Total oxyradical scavenging capacity

The total oxyradical scavenging capacity assay is based on the reaction between artificially generated oxyradicals and α -keto- γ -(methylthio)butyric acid (KMBA), which is oxidized to ethylene. The capability of a sample to scavenge oxyradicals is quantified from its ability to inhibit ethylene formation relative

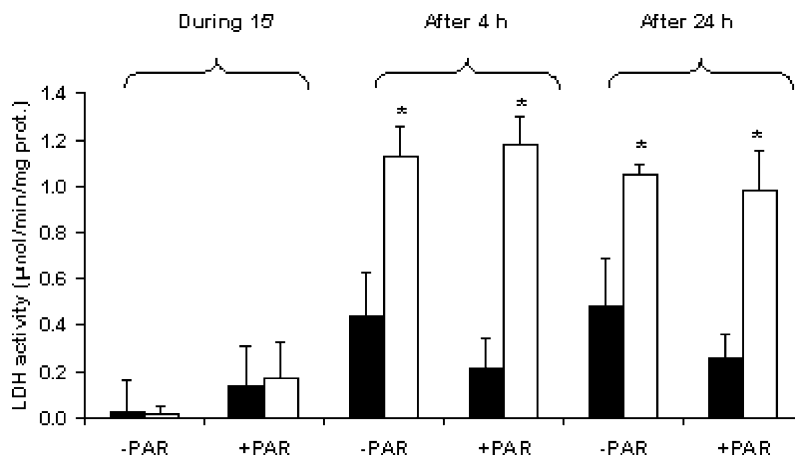


Fig. 5. LDH activity in supernatant of cells exposed to 15 min of UVA in the absence (–PAR) or presence (+PAR) of 10 μM Parsol 1789 during 15 min of exposure and at 4 and 24 h post-UVA illumination. Black bars correspond to non-illuminated cells whereas white bars correspond to illuminated cells. LDH activity is expressed as μmol of pyruvate consumed/min/mg proteins obtained in the cell population (mean values \pm S.D. $n = 3$). The differences between non-illuminated and illuminated samples after 4 and 24 h post-illumination are always statistically significant whether in the presence or absence of Parsol 1789 (* $P < 0.01$).

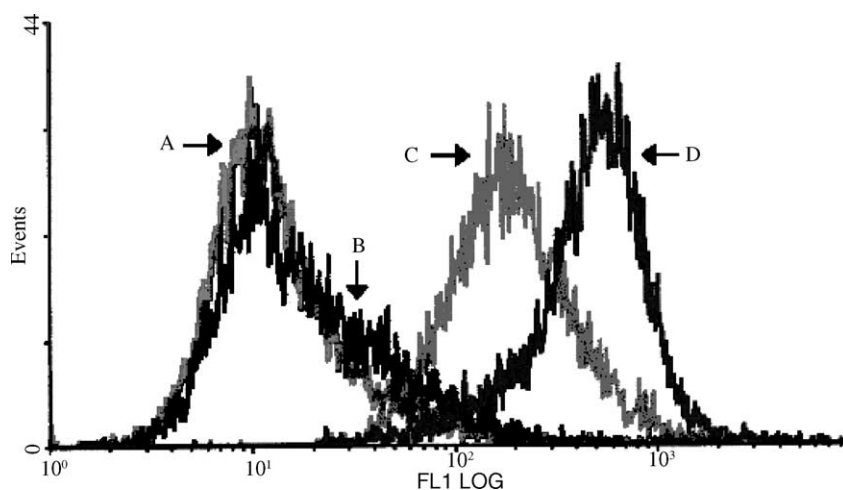


Fig. 6. The figure shows the fluorescence intensity of annexin V in cells non-illuminated or illuminated in the presence or absence of $10 \mu\text{M}$ Parsol 1789. Non-illuminated cells without Parsol 1789 (A); non-illuminated cells treated with Parsol 1789 (B); illuminated cells without Parsol 1789 (C); and illuminated cells treated with Parsol 1789 (D). Cells were analysed as described in Section 2 after 24 h post-UVA illumination. The histograms are representative of at least three individual experiments. The peak position of the annexin V staining is 1.01 ± 0.06 for A, 1.05 ± 0.08 for B, 15.3 ± 1.7 for C and 53.6 ± 6.8 for D.

to a control reaction containing no biological sample (Winston et al., 1998). Two oxidant-generating systems designed to produce independently peroxy radicals and hydroxyl radical were used to assess the total antioxidant scavenging capacity of NCTC2544

keratinocytes when exposed to UVA in the absence or presence of Parsol 1789 after 4 and 24 h post-illumination.

Fig. 7 shows that exposure to UVA induced after 4 h, a significant increase in total oxyradical scavenging

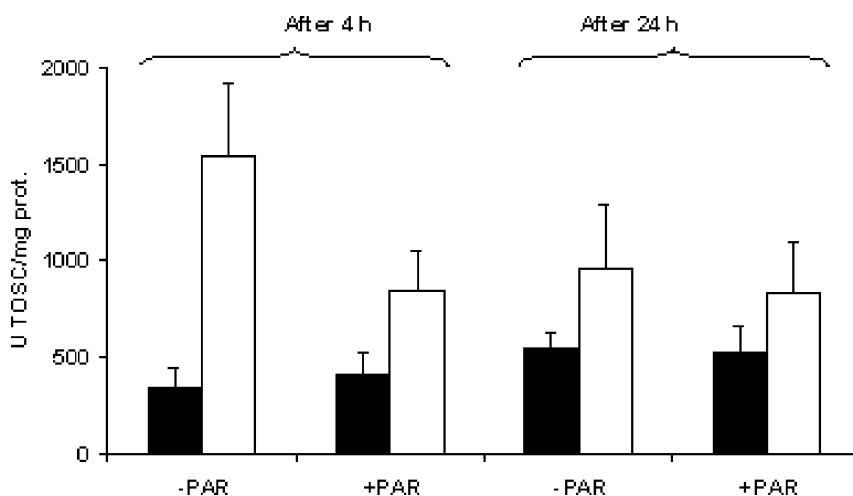


Fig. 7. Total oxyradical scavenging capacity toward peroxy radicals generated by AAPH measured in cultured keratinocytes exposed to 15 min of UVA in the absence (-PAR) or presence (+PAR) of $10 \mu\text{M}$ Parsol 1789 at 4 and 24 h post-UVA illumination. Black bars correspond to non-illuminated cells whereas white bars correspond to illuminated cells. The values are expressed as TOSC units/mg proteins (mean values \pm S.D. $n = 4$). Significant differences from the control were statistically significant whether in the presence or absence of Parsol 1789 after 4 h ($*P < 0.01$).

capacity toward peroxy radicals generated by AAPH, which drops after 24 h. This induction is less remarkable when the cells were treated and illuminated with Parsol 1789 both at 4 and 24 h from exposure.

The total oxyradical scavenging capacity toward hydroxyl radicals generated by the iron/ascorbate system is reported in Fig. 8. In this case too, after 4 h from exposure to UVA, there is a notable increase in TOSC values with respect to those not exposed which decreases dramatically after 24 h. The cells exposed to UVA and treated with Parsol 1789 also show an increase in TOSC values but not as remarkable as the control cells exposed to UVA.

3.4. Total ROS production

The use of fluorescent probes is a popular method to detect a broad range of cell-derived oxidants that may be increased during intracellular oxidative stress (Hempel et al., 1999). Carboxy-H₂DCFDA which is non-fluorescent, readily enters cells, is cleaved by esterases, and emits fluorescence when it is oxidised to dichlorofluorescein. By quantifying the fluorescence, an overall oxidative status of the cell is obtained. Fig. 9A shows that there is a significant increase in

fluorescence in cells right after exposure to UVA, and that the presence of Parsol 1789 has no positive nor negative effect on this increase. Interestingly, in Fig. 9B when GSH is present alone in illuminated cells, the fluorescence emitted is almost reduced to the control levels of non-illuminated cells; however, this positive effect due to GSH is cancelled when Parsol 1789 is also present in the incubation medium.

3.5. Lipid peroxidation

Measurement of lipid hydroperoxides using the FOX2 method is a convenient, sensitive and simple means for quantifying lipid soluble hydroperoxides in biological samples, such as those present in lipoproteins, membranes and fats formed during the oxidative event of lipid peroxidation. By using this assay, the results obtained in Table 1 show that the level of hydroperoxides detected in samples straight after exposure to UVA in the absence of Parsol 1789 increases more than two-fold compared to the non-illuminated controls. When the sunscreen is present, this increase in hydroperoxides is remarkably higher. Glutathione seems to have a protective effect, even if not significant, on cells exposed to UVA in the absence of

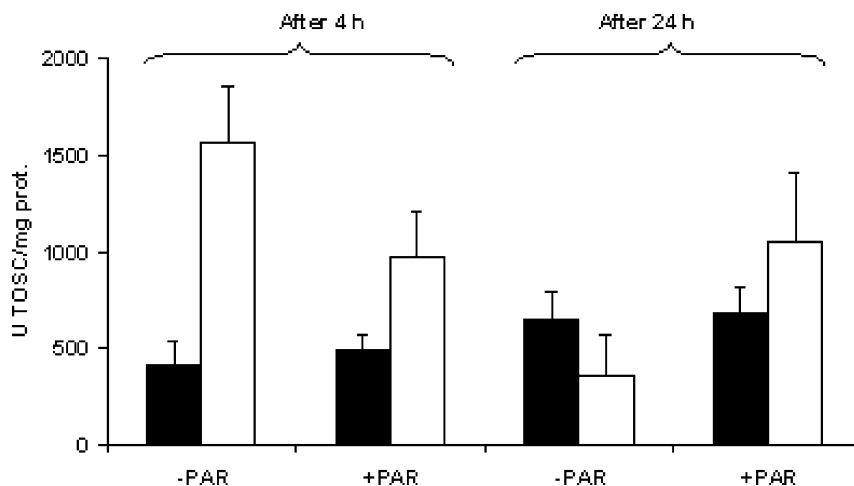


Fig. 8. Total oxyradical scavenging capacity toward hydroxyl radicals generated by Fe³⁺/ascorbate system, measured in cultured keratinocytes exposed to 15 min of UVA in the absence (–PAR) or presence (+PAR) of 10 μM Parsol 1789 at 4 and 24 h post-UVA illumination. Black bars correspond to non-illuminated cells whereas white bars correspond to illuminated cells. The values are expressed as TOSC units/mg proteins (mean values ± S.D. *n* = 4). Significant differences from the control were statistically significant whether in the presence or absence of Parsol 1789 after 4 h (**P* < 0.01).

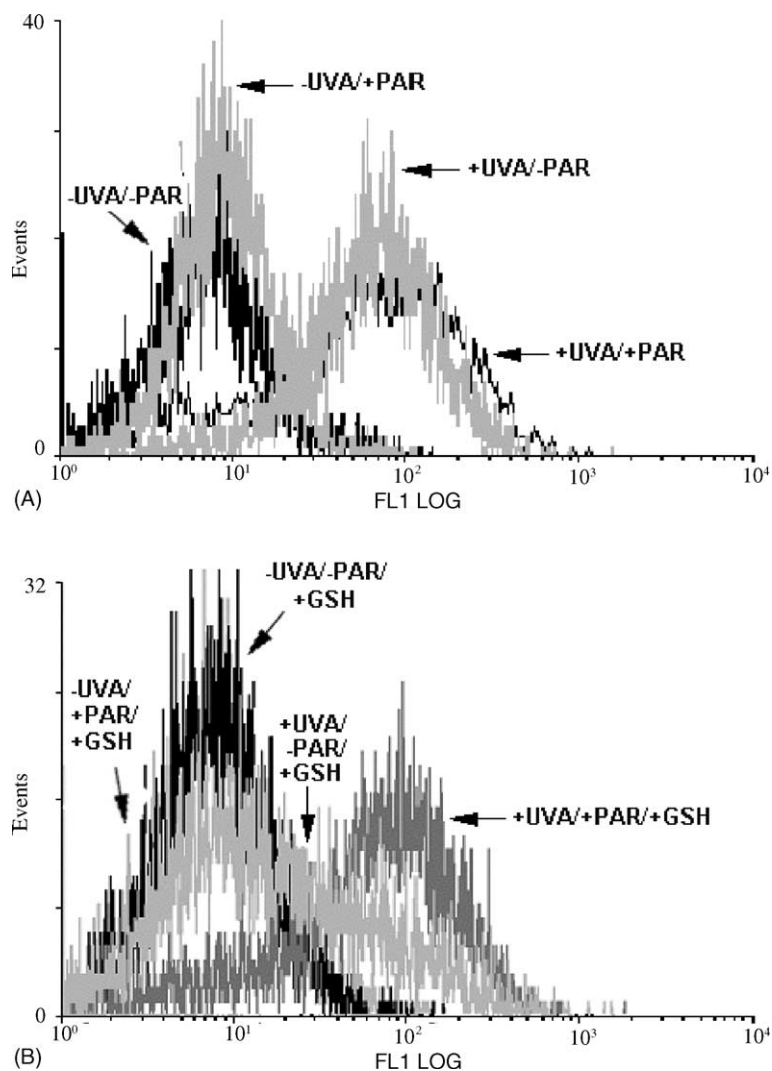


Fig. 9. The figure shows the fluorescence intensity of carboxy- H_2DCFDA in cells non-illuminated or illuminated in the presence or absence of $10\mu M$ Parsol 1789 (A) and/or 1 mM GSH (B). The histograms are representative of at least three individual experiments. In (A), the peak position of the carboxy- H_2DCFDA fluorescence is 0.702 ± 0.93 for $-UVA/-PAR$, 0.911 ± 1.25 for $-UVA/+PAR$, 5.92 ± 1.25 for $+UVA/-PAR$, 6.96 ± 2.93 for $+UVA/+PAR$. In (B), the peak position of the carboxy- H_2DCFDA fluorescence is 0.712 ± 0.74 for $-UVA/+GSH/-PAR$, 1.12 ± 0.87 for $-UVA/+GSH/+PAR$, 1.15 ± 0.59 for $+UVA/+GSH/-PAR$, 10.5 ± 1.53 for $+UVA/+GSH/+PAR$. Cells were analysed as described in Section 2 straight after UVA exposure. The histograms are representative of at least three individual experiments.

Parsol 1789, but when present in combination with the sunscreen, this protective effect is reduced. After 4 h from exposure, all the values measured in the illuminated cells tend to normalize themselves although slightly higher values are always registered in sunscreen-treated samples.

4. Discussion

UV-protection means conversion of the radiation energy of sunlight into a harmless form. Efficient UV-absorbers used in cosmetics and toiletries must therefore be chemically and photochemically inert.

If they are not, chemical bonds may be rearranged leading to new molecules, the absorbance of which might be diminished or even lost, and the toxicological properties may be altered (Tarras-Wahlberg et al., 1999). Reactive intermediates such as free radicals may be produced which lead to biological damage. Therefore, the light energy absorbed by UV-filters (if not scattered or reflected) should only be transformed into harmless thermal energy. Unfortunately, this is not the case for the most efficient UVA filter widely present on the market in sun care products since 1983, 4-*tert*-butyl-4'-methoxydibenzoylmethane (commercially known as Parsol 1789 or Avobenzone) (De Polo, 1980) and approved by regulatory authorities of Europe, USA, Japan and Australia (EEC Directive, 2004; US FDA, 1999; Hayden et al., 1998). Although it covers almost the entire UVA range, this compound is inherently unstable: after only 1 h of illumination of a very thin film layer of an emulsion of Parsol 1789, degradation rates up to 50% were found (Deflandre and Lang, 1988). In fact, Parsol 1789 initially breaks down to two carbon-centered free radicals (benzoyl and phenacyl; Fig. 1) which subsequently rearrange and react to give an array of products (Schwack and Rudolf, 1995). This is probably the reason for the non-protective effects of this compound observed in several biological systems (Damiani et al., 1999, 2000; Tran et al., 2002). The results now obtained in the present investigation extend further the information on the lack of photoprotection of Parsol 1789.

From the results reported in Figs. 2–4, it is clear that exposure of keratinocytes to 15 min UVA radiation leads to substantial cell mortality and decrease in protein content, which is particularly noticeable after 24 h from exposure with respect to control cells and that the presence of 10 μ M Parsol 1789 has no protective effect on these events. It is documented that one of the effects of UVA-induced cytotoxicity in keratinocytes is the release of LDH (Saliou et al., 1999; Fu et al., 2000) therefore this parameter was also determined to see if Parsol 1789 affects this event associated with membrane damage and leakage. No significant differences in LDH release was observed between Parsol 1789-treated and untreated illuminated cells at after all times from exposure (Fig. 5) indicating that even in this case, no protection is conferred by the sun filter.

It has now been well established that UVA exposure induces a variety of toxic and modulating events which cause suicidal cell death (Tada-Oikawa et al., 2000). Both Hu et al. (2003) and Fu et al. (2000) found that UVA irradiation of keratinocytes caused an increase in the percentage of apoptosis and their results agree with the findings observed in the present investigation. However, the presence of this UVA-absorber further increments this cellular event. The increased apoptosis in illuminated keratinocytes in the presence of Parsol 1789 is an interesting finding as it suggests that the potential of this compound to invoke inflammation or neoplastic transformation is minimized.

Besides the induction of apoptosis in cells, it is well known that UVA irradiation also induces oxidative damage at the molecular and cellular level caused by both direct and indirect free radical generation, and it is believed that it is these reactive oxygen species which are responsible for photoaging of human skin (Vile and Tyrell, 1995; Scharffetter-Kochanek et al., 1997; Herrling et al., 2002; Dalle Carbonare and Pathak, 1992). Fortunately, a plethora of interlinked antioxidant defense mechanisms exist in mammalian cells to counteract this UV radical-induced oxidative damage (Shindo et al., 1994; Fuchs, 1998). These range from the expression of antioxidant enzymes such as superoxide dismutase, catalase and several peroxidases as well as compounds which quench reactive oxygen species, such as ascorbate, uric acid, carotenoids and glutathione. From the results reported in Figs. 7 and 8, where the total oxyradical scavenging capacity of human keratinocytes towards peroxy and hydroxyl radicals was evaluated before and after UV exposure, it can be deduced that UVA exposure remarkably enhances the antioxidant defence mechanisms in keratinocytes, especially after 4 h post-illumination. The defensive mechanisms then drop to almost normal levels after 24 h post-illumination. In fact, keratinocytes may respond to the UV exposure by up-regulating antioxidant enzymes as has already been observed in cultured fibroblasts where increased Mn-SOD and glutathione peroxidase were detected on increasing doses of UVA exposure (Meewes et al., 2001). TOSC values also increase in illuminated cells containing Parsol 1789 but to a lesser extent than the control cells. This finding might imply two things: either that the presence of this UV-absorber in some way interferes with the antioxidant defense system or

that Parsol 1789 being it a UVA filter, decreases the received UVA dose, and as a consequence, the biological events induced by UVA will also be lower. This latter could be regarded as a positive action. However, since the other findings obtained in this work show that Parsol 1789 does not protect these cells under illumination, it is more probable that the photo-decomposition products of Parsol 1789 which include free radicals, may directly or indirectly damage and inactivate the antioxidant defence system. For example, antioxidant proteins may be damaged and this may be supported by the fact that Parsol 1789 has been previously observed *in vitro* to increase oxidative modifications in a protein model (Damiani et al., 2000). In addition, Dalle Carbonare and Pathak (1992) have shown that protein inactivation is more significant by UVA irradiation in the presence of sensitizers than by UVA without sensitizers. Gulston and Knowland (1999) have demonstrated that another UV filter, 2-ethylhexyl-4-dimethylaminobenzoate (Padimate-O) in contact with basal keratinocytes, increases DNA strand breaks when exposed to UV radiation and has been shown to be mutagenic to yeast (Knowland et al., 1993). The widely used UVB sunscreen, 2-phenylbenzimidazole-5-sulfonic acid, also sensitizes the production of singlet oxygen and photo-induces the formation of alkali-labile cleavage sites in both single- and double-stranded DNA (Stevenson and Davies, 1999). Further evidence comes from the results obtained in Fig. 9B and Table 1. When the antioxidant glutathione alone is present in the medium and cells are exposed to UVA, an overall decrease in oxidative stress is detected (Fig. 9B) reaching almost the level of non-illuminated control cells (Fig. 9A), whereas when Parsol 1789 alone is added, this decrease in UVA-induced oxidative stress (Fig. 9A) does not occur. However, when both compounds are present together in contact with illuminated keratinocytes the positive effect observed with GSH is nullified suggesting that in some way, the antioxidant is most likely consumed by interacting with the radicals directly or indirectly produced through photo-decomposition of Parsol 1789 in the incubation medium. The level of lipid hydroperoxides detected in illuminated cells straight after exposure also increases in comparison to control cells, due to UVA-induced lipid peroxidation, and this event is further exacerbated when the sunscreen is present (Table 1). The

simultaneous presence of GSH leads only to a partial reduction in lipid peroxidation, again suggesting that it is consumed by the oxidative events caused by the presence of Parsol 1789. After 4 h from exposure, the level of hydroperoxides decreases most probably due to the induction of repair mechanisms of lipid peroxidation such as cleavage and/or removal of lipid hydroperoxides by phospholipases and glutathione peroxidase.

The overall conclusion that can now be drawn from the investigation of the effects of this UVA-absorber is that under the experimental conditions employed in this study, Parsol 1789 in contact with keratinocytes does not protect them from UVA-induced cytotoxicity and oxidative stress. Other UVA absorbers, for example Mexoryl SX, have instead been found to protect keratinocytes when in contact with them under solar simulated UV light (Marrot et al., 1998). The present study also confirms our previous findings on the effects of Parsol 1789 which indicated that although this compound is designed to filter out UVA light, it does not photoprotect relevant biological molecules against direct UVA damage when in contact with them. Future work is now addressed to finding out what its effects are at the molecular level in cells and the mechanisms involved to explain the results observed in the present study. Because filter molecules may penetrate the epidermal structures and highly reactive intermediates of photounstable filter substances get in direct contact with epidermal (Gulston and Knowland, 1999; Jiang et al., 1999; Hayden et al., 1997) and dermal structures (Schallreuter et al., 1996), the results presented in this paper on the effects of Parsol 1789 on cultured human keratinocytes may be of significance, especially if one considers that the UVA-filter effect of the sunscreen will be strongly reduced when illuminated as it photo-decomposes. Hence, the UVA dose penetrating the living part of the epidermis will not be that negligible. However, it is important to always underline that the biological role and significance of this investigation to the consequences of sunscreen use in humans are not known and extrapolation from laboratory experiments must be done with caution. Photodamage is predicted to become a major threat to public health in the coming decades therefore, assuming that sunscreens have some role to play in preventing skin photodamage and cancer, studies on their

effectiveness, stability, delivery, toxicity and safety should be increased.

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