The Sunscreen Agent 2-Phenylbenzimidazole-5-Sulfonic Acid Photosensitizes the Formation of Oxidized Guanines *In Cellulo* after UV-A or UV-B Exposure

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The sunscreen agent 2-phenylbenzimidazole-5-sulfonic acid (PBSA) is water soluble and is widely used in the cosmetic industry because it absorbs strongly at UV-B wavelengths. Previous studies have shown that PBSA, photoexcited by UV-B, oxidizes guanine bases *in vitro*. Because of its potential phototoxic effect, it is important to determine whether PBSA photosensitizes *in cellulo* the formation of oxidatively generated DNA damage on UV exposure. For this purpose, we investigated, *in vitro* and *in cellulo*, the effect of PBSA on DNA bases after UV-A or UV-B irradiation. To monitor the formation of oxidized bases and cyclobutane pyrimidine dimers (CPDs), DNA was digested either with FaPy-DNA glycosylase and endonuclease III or with T4 endonuclease V and photolyase, then analyzed by means of neutral- and glyoxal-agarose gel electrophoresis and ligation-mediated PCR. *In cellulo*, we found that PBSA provided good protection against CPD formation after UV-B exposure. However, PBSA also photosensitized oxidized guanines on UV-A and UV-B irradiation. Our results indicate that PBSA has the potential to function as a double-edged sword toward DNA and question its suitability for sunscreen applications.

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INTRODUCTION

It is a well-known fact that extensive sunlight exposure is hazardous to human health and can lead to outcomes that include erythema, skin aging, immunosuppression, and skin cancers (Fisher *et al.*, 1996; Sarasin, 1999; Schwarz, 2005). Sun-related skin cancers are a major concern, as the World Health Organization estimates that 54,000 people worldwide die each year from cancers due to UV light. Prevention is mainly promoted by encouraging the use of sunscreens and by reducing sunlight exposure. The principle of sunscreens is to block UV-B (290–320 nm) and UV-A (320–400 nm) through the action of inorganic or organic molecules that respectively reflect or absorb these high-energy UV photons. The efficacy of sunscreens against sunburn is proven, as they block UV-B, which is responsible for erythemal reaction. However, their protective effect against melanoma and basal cell carcinoma is still under debate, as not all sunscreens block UV-A efficiently (Gasparro, 2000) and their use generally leads to intentionally extended sun exposure (Autier *et al.*, 2007). Moreover, several studies have shown that some sunscreen agents function as photosensitizers (Allen *et al.*, 1996a, b; Xu *et al.*, 2001; Hanson *et al.*, 2006).

2-Phenylbenzimidazole-5-sulfonic acid (PBSA), also called ensulizole, is a molecule approved by the U.S. Food and Drug Administration, which is often found in cosmetic sunscreen formulations because of its intense absorption at UV-B wavelengths and its high solubility in water. Stevenson and Davies (1999) showed that UV-B irradiation of DNA *in vitro* in the presence of PBSA leads to the formation of oxidized guanines by both type I and type II photosensitization mechanisms. These findings were reinforced by the photophysical and photochemical characterization of PBSA by Inbaraj *et al.* (2002), who clearly demonstrated the capacity of PBSA to generate reactive oxygen species, including singlet oxygen, on photoexcitation. Altogether, these data suggest that, in the cellular context, PBSA could possibly damage DNA, as well as proteins and lipids, through a photosensitizing mechanism.

The aim of this study was to investigate, *in vitro* and *in cellulo*, the effect of PBSA on DNA after UV-A or UV-B irradiation. For this purpose, purified DNA or fibroblasts were irradiated using specific doses providing similar damage frequencies in the presence of millimolar concentrations of PBSA. To monitor the formation of oxidized

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Abbreviations: CPD, cyclobutane pyrimidine dimer; DMEM, Dulbecco's modified Eagle Medium; DSB, double-strand break; Endo III, endonuclease III; Fpg, FaPy-DNA glycosylase; LMPCR, ligation-mediated PCR; ox G, oxidized guanine; 8-oxoGua, 8-oxo-7,8-dihydroguanine; PBSA, 2-phenylbenzimidazole-5-sulfonic acid; SB, strand break; SSB, single-strand break; T4 endo V, T4 endonuclease V

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bases and cyclobutane pyrimidine dimers (CPDs), DNA was digested with either FaPy-DNA glycosylase and endonuclease III (Fpg + Endo III) or T4 endonuclease V and photolyase (T4 endo V + photolyase), then analyzed by neutral- and glyoxal-agarose gel electrophoresis and ligation-mediated PCR (LMPCR) (Pfeifer *et al.*, 1993; Drouin *et al.*, 1996b).

We found that, *in cellulo*, PBSA protected against CPD formation after UV-B exposure but photosensitized the formation of oxidized guanine after UV-A or UV-B irradiation. Our results suggest that this sunscreen agent could potentially function as a double-edged sword toward DNA.

RESULTS

In vitro effect of PBSA on purified DNA irradiated with UV-A or UV-B

In a first approach, we studied how the presence of PBSA (0–4 mM) affected the photoreactivity of purified fibroblast DNA exposed to UV-B or UV-A radiations *in vitro*.

Purified DNA was exposed to 50 kJ m^{-2} UV-B, and CPD formation was measured by digesting DNA with the combined action of T4 endo V + photolyase to convert CPD into strand breaks. The generated DNA fragments were then fractionated according to their size on neutral- and glyoxal-agarose gels, after which DNA strand break frequency was calculated (Figure 1a and b; see Supplementary Figure S1a and b online). It is worth noting that both double-strand breaks (DSBs) and single-strand breaks (SSBs) are measured using glyoxal gel, whereas neutral gel only assesses DSB. Interestingly, similar results were obtained using neutral and glyoxal gels. In both cases, when DNA was irradiated with UV-B in the absence of PBSA, CPDs were readily produced (Figure 1a and b, Supplementary Figure S1a-b lanes 1). When PBSA was added, CPD formation was reduced as PBSA concentration increased (Figure 1a and b, Supplementary Figure S1a-b lanes 2–4). Using LMPCR, we observed that two phenomena occurred (Figure 1c). As PBSA concentration increased, the amount of CPDs decreased, with CPD formation totally prevented at 4 mM PBSA. However, an increase in PBSA concentration was also correlated with an increase in damage at guanine sites. Indeed, samples were treated with T4 endo V + photolyase to monitor for CPDs and not with Fpg + Endo III; thus, the bands observed at guanine sites must be strand breaks.

Oxidatively generated lesions were monitored after 50 kJ m^{-2} UV-B irradiation. To determine which bases were oxidized, we used Fpg + Endo III, which converts oxidized purines and oxidized pyrimidines into strand breaks. In the absence of Fpg + Endo III treatment, strand breaks were observed with glyoxal gel (Figure 1d and e, Supplementary Figure S1c-d lanes 9–10). However, Fpg + Endo III-treated samples gave similar results using neutral and glyoxal gels. We observed oxidized bases as PBSA concentration increased (Figure 1d and e, Supplementary Figure S1c–d lanes 4). When analyzed by LMPCR, base oxidation occurred at guanine sites and was prominently induced with 4 mM PBSA (Figure 1f). Neither oxidized adenine nor pyrimidine bases were measured.

DNA was irradiated with 250 kJm-2 UV-A in the presence of PBSA, and CPDs were monitored. DNA strand breaks were observed in the absence of T4 endo V + photolyase treatment with 4 mM PBSA with glyoxal gel, but not with neutral gel (Figure 2a and b, Supplementary Figure S2a-b). By consequence, these strand breaks were most likely SSB. In the presence of T4 endo V + photolyase, the average strand break frequency was globally augmented. This suggests that UV-A induced CPD formation (Figure 2a and b, Supplementary Figure S2a-b). To determine at which base DNA damage was formed, we performed LMPCR (Figure 2c). Clearly, SSBs were formed at guanine sites as PBSA concentration increased. However, the sensitivity of the LMPCR technique was not sufficiently high to clearly observe CPD (data not shown). Indeed, we showed in a former study that much higher doses are needed to monitor CPDs by LMPCR after UV-A irradiation (Rochette *et al.*, 2003).

We then measured oxidized bases using the same irradiation protocol, but irradiated DNA was treated with Fpg + Endo III. By performing neutral gel electrophoresis, we observed that, in the absence of Fpg + Endo III treatment, few DSBs were formed at the highest PBSA concentration (Figure 2d, Supplementary Figure S2c lane 10). Treatment with Fpg + Endo III led to fragmented DNA as PBSA concentration increased, indicating that bases were oxidized by photo-activated PBSA. Analysis of these samples by glyoxal gel leads to identical observations. However, it is important to note that in the case of Fpg + Endo III nontreated samples, an important amount of SSBs were generated with 4 mM PBSA (Figure 2e, Supplementary Figure S2d lane 10). To determine which bases were oxidized, we performed LMPCR (Figure 2f). Clearly, PBSA induced damage at guanine sites as concentration increased and no damage was observed on other bases. Analysis of samples that were only digested by Fpg led to the same results using LMPCR (Supplementary Figure S3). We propose that two types of damage were formed *in vitro* after UV-A. First, at high concentration, PBSA is capable of introducing DNA strand breaks, as observed in the absence of damage conversion on gels. Second, even at low concentrations, PBSA oxidizes guanines.

Altogether, *in vitro* results clearly show that PBSA reduces CPD formation after UV-B, even at a concentration as low as 0.125 mM. However, although it protects from UV-B-induced CPD, it photosensitizes oxidized guanines, confirming the observations made by Stevenson and Davies (1999). Moreover, we observed that PBSA photosensitizes guanines after UV-A irradiation as well. Type I photosensitization signature is the preferential oxidation of 5′-guanine in 5′-GG-3′ doublets (Hiraku *et al.*, 2007). As there was no asymmetrical oxidation of guanine *in vitro*, type II photosensitization must operate, but we could not arrive at a conclusion with regard to type I photosensitization. In addition to oxidized guanines, PBSA photosensitized strand breaks after UV-A and UV-B. To determine whether SSBs and DSBs were formed at guanine sites for all *in vitro* conditions, we performed LMPCR of Fpg + Endo III or T4 endo V + photolyase nontreated samples. We observed

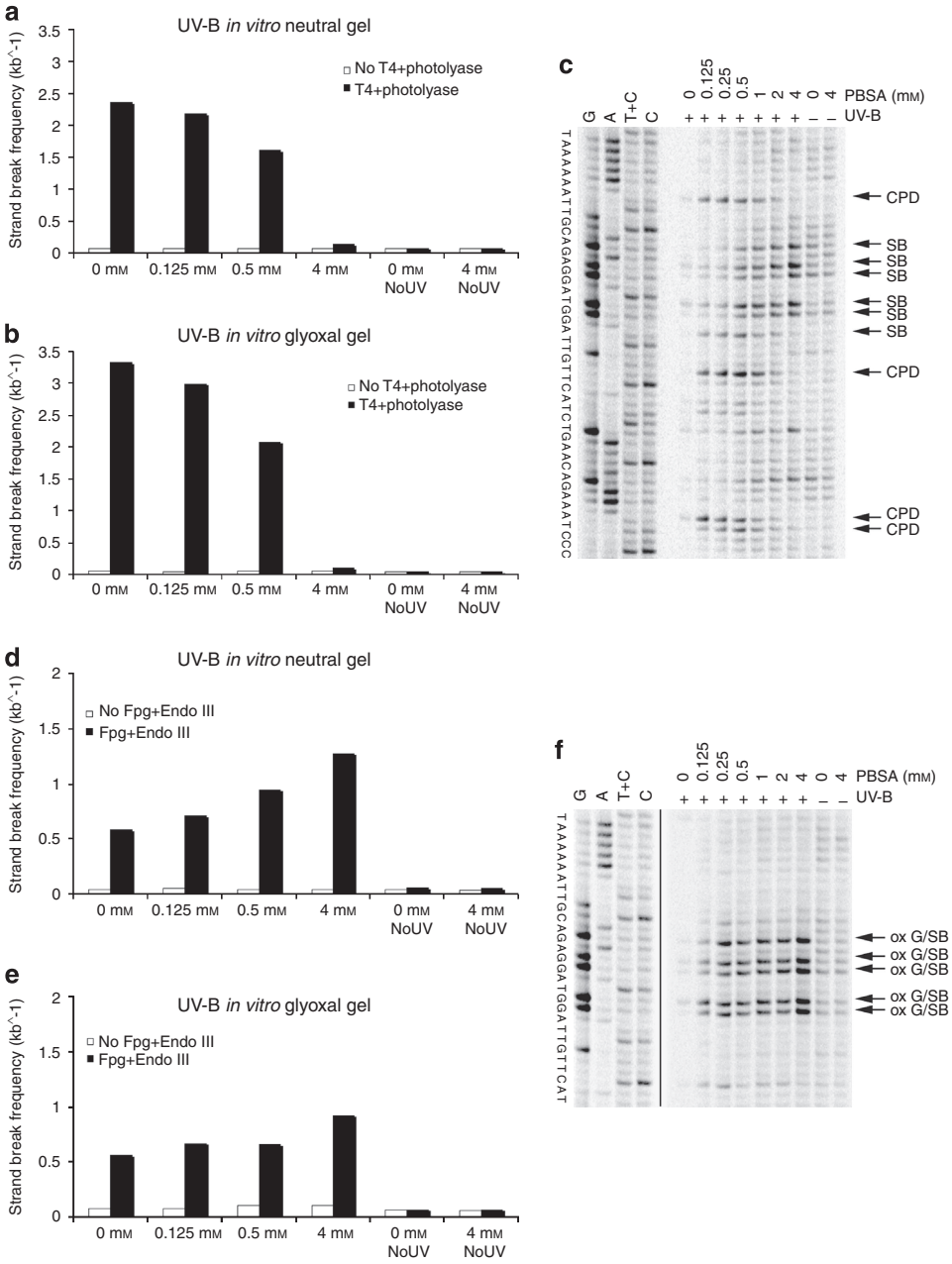

Figure 1. Effect of 2-phenylbenzimidazole-5-sulfonic acid (PBSA) on the formation of cyclobutane pyrimidine dimers and oxidized bases in isolated DNA irradiated with UV-B. Purified fibroblast DNA was irradiated with 50 kJ m^{-2} UV-B in the presence of PBSA (0–4 mM); thereafter, DNA was digested or not with T4 endonuclease V and photolyase (T4 endo V + photolyase). DNA strand break frequency was analyzed by neutral-agarose gel (**a**) and glyoxal-agarose gel (**b**) and then quantified. (**c**) DNA damage cartography on the nontranscribed strand of *p21*. Samples digested by T4 endo V + photolyase were analyzed by ligation-mediated PCR; the sequencing gel is presented. DNA was digested or not digested with FaPy-DNA glycosylase and endonuclease III (Fpg + Endo III). DNA strand break frequency was analyzed by neutral-agarose gel (**d**) and glyoxal-agarose gel (**e**) and then quantified. (**f**) DNA damage cartography on the nontranscribed strand of *p21*. Samples digested by Fpg + Endo III were analyzed by ligation-mediated PCR; the sequencing gel is presented. Black line indicates that samples were not run consecutively on the gel.

that SSBs and DSBs were always generated at guanine sites (data not shown).

Effect of PBSA *in cellulo* after UV-A or UV-B irradiation

We then examined the effects of PBSA *in cellulo* using the same experimental approach that we used for *in vitro* investigation. Human normal primary fibroblasts were irradiated with either UV-B or UV-A, to determine the role of each radiation in the PBSA phototoxicity.

To assess the effect of UV-B on PBSA phototoxicity, human primary fibroblasts were irradiated with 50 kJ m^{-2} UV-B in the presence of PBSA (0–4 mM). Neutral and glyoxal gels led to identical observations. In the absence of CPD conversion by T4 endo V + photolyase treatment, no

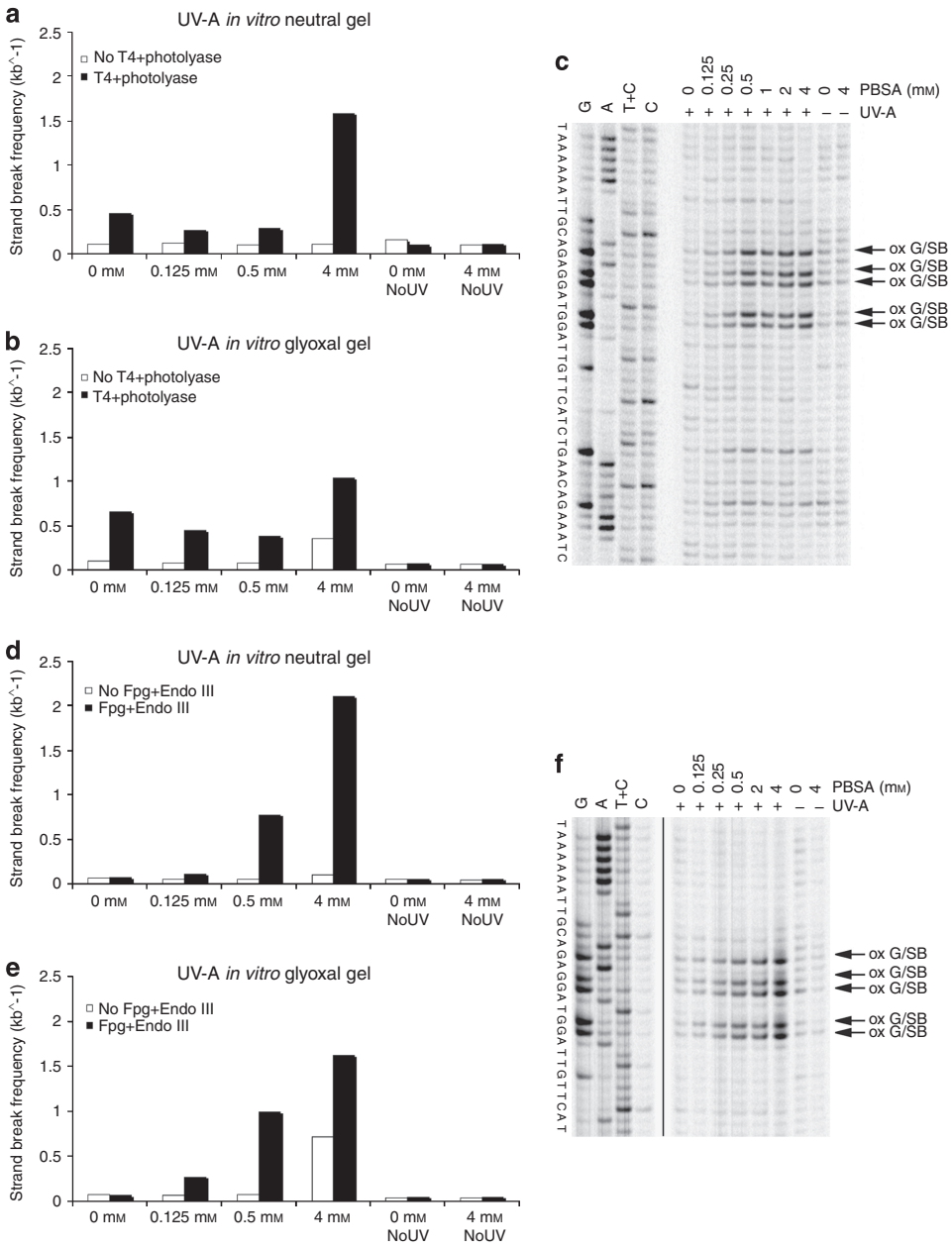

Figure 2. Effect of 2-phenylbenzimidazole-5-sulfonic acid (PBSA) on the formation of cyclobutane pyrimidine dimers and oxidized bases in isolated DNA irradiated with UV-A. Purified fibroblast DNA was irradiated with 250 kJ m^{-2} UV-A in the presence of PBSA (0–4 mM); thereafter, DNA was digested or not with T4 endonuclease V and photolyase (T4 endo V + photolyase). DNA strand break frequency was analyzed by neutral-agarose gel (**a**) and glyoxal-agarose gel (**b**) and then quantified. (**c**) DNA damage cartography on the nontranscribed strand of *p21*. Samples digested by T4 endo V + photolyase were analyzed by ligation-mediated PCR; the sequencing gel is presented. DNA was digested or not with FaPy-DNA glycosylase and endonuclease III (Fpg + Endo III). DNA strand break frequency was analyzed by neutral-agarose gel (**d**) and glyoxal-agarose gel (**e**) and then quantified. (**f**) DNA damage cartography on the nontranscribed strand of *p21*. Samples digested by Fpg + Endo III were analyzed by ligation-mediated PCR; the sequencing gel is presented. Black line indicates that samples were not run consecutively on the gel.

strand breaks were detected (Figure 3a and b, Supplementary Figure S4a-b lanes 7–12). When T4 endo V + photolyase treatment was performed, CPD formation was clearly evident when no PBSA was present. When PBSA was added, CPD formation decreased, as we previously observed *in vitro* (Figure 3a and b, Supplementary Figure S4a-b lanes 1–6). These data were corroborated by LMPCR results. They clearly show a PBSA-dependent protection against CPD (Figure 3c). However, 4 mM PBSA was not sufficient enough to totally protect against CPD (Figure 3a and b, Supplementary Figure S4a-b lane 4; Figure 3c).

When oxidatively generated damage was monitored by neutral and glyoxal gel in the absence of Fpg + Endo III treatment, neither SSB nor DSB were found to be induced by

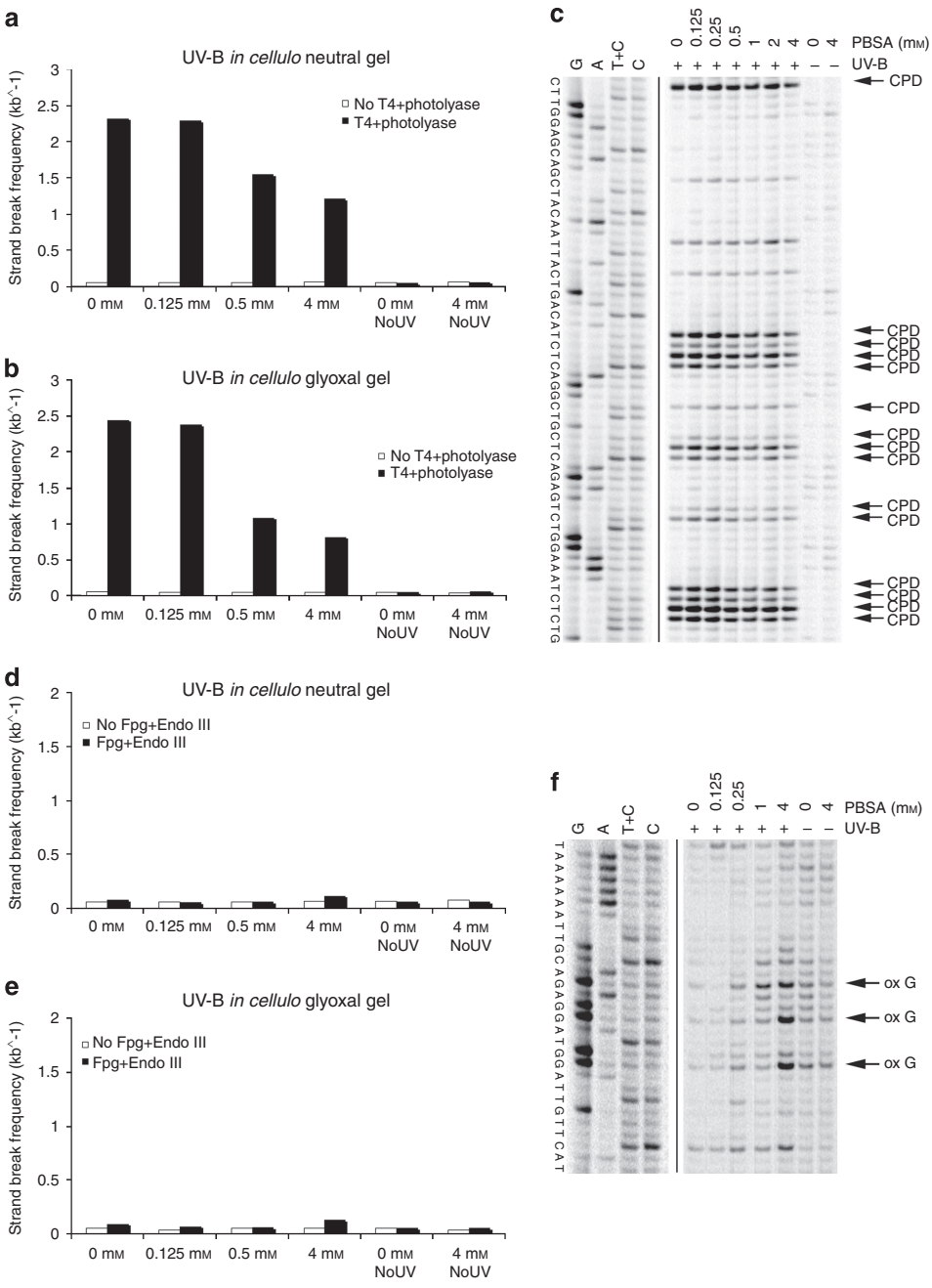

Figure 3. Effect of 2-phenylbenzimidazole-5-sulfonic acid (PBSA) on the formation of cyclobutane pyrimidine dimers and oxidized bases in human primary fibroblasts irradiated with UV-B. Human primary fibroblasts were irradiated with 50 kJ m^{-12} UV-B in the presence of PBSA (0–4 mM); thereafter, DNA was digested or not with T4 endonuclease V and photolyase (T4 endo V + photolyase). DNA strand break frequency was analyzed by neutral-agarose gel (**a**) and glyoxal-agarose gel (**b**) and then quantified. (**c**) DNA damage cartography on the nontranscribed strand of *p21*. Samples digested by T4 endo V + photolyase were analyzed by ligation-mediated PCR; the sequencing gel is presented. DNA was digested or not with FaPy-DNA glycosylase and endonuclease III (Fpg + Endo III). DNA strand break frequency was analyzed by neutral-agarose gel (**d**) and glyoxal-agarose gel (**e**) and then quantified. (**f**) DNA damage cartography on the nontranscribed strand of *p21*. Samples digested by Fpg + Endo III were analyzed by ligation-mediated PCR; the sequencing gel is presented. Black lines indicate that samples were not run consecutively on the gel.

PBSA after UV-B irradiation (Figure 3d and e, Supplementary Figure S4c-d lanes 7–12). Samples treated with Fpg + Endo III revealed that only 4 mM PBSA generated oxidized bases (Figure 3d and e, Supplementary Figure S4c-d lane 4). LMPCR results show that only oxidized guanines were photosensitized by PBSA in a concentration-dependent manner (Figure 3f). Interestingly, the photosensitizing effect only occurred at PBSA concentrations superior to 0.5 mM, which is higher to that observed *in vitro*.

To assess the contribution of UV-A on PBSA phototoxicity *in cellulo*, we irradiated human primary fibroblasts with 250 kJ m^{-2} UV-A in the presence of PBSA (0–4 mM).

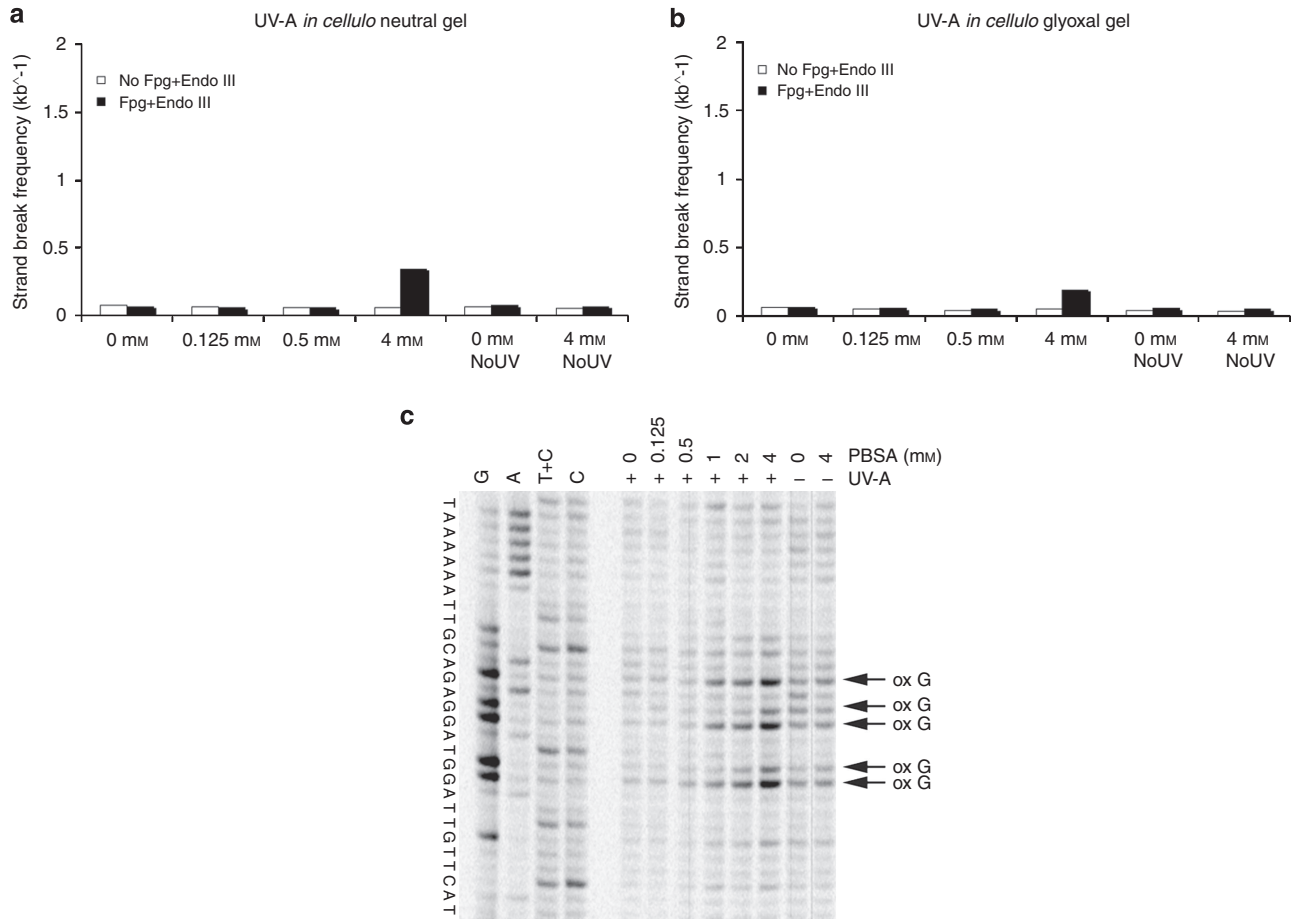

Figure 4. Effect of 2-phenylbenzimidazole-5-sulfonic acid (PBSA) on the formation of oxidized bases in human primary fibroblasts irradiated with UV-A. Human primary fibroblasts were irradiated with 250 kJ m^{-2} UV-A in the presence of PBSA (0–4 mM); thereafter, DNA was digested or not with FaPy-DNA glycosylase and endonuclease III (Fpg + Endo III). DNA strand break frequency was analyzed by neutral-agarose gel (**a**) and glyoxal-agarose gel (**b**) and then quantified. DNA damage cartography on the nontranscribed strand of *p21*. Samples digested by Fpg + Endo III were analyzed by ligation-mediated PCR; the sequencing gel is presented (**c**).

We measured CPDs by using neutral and glyoxal gels, as well as LMPCR combined with T4 endo V + photolyase treatment of samples. We did not observe any damage by any of these methods (data not shown).

We then measured oxidatively generated damage formation by digesting DNA with Fpg + Endo III. Analysis of nondigested samples using neutral and glyoxal gels showed that PBSA induced neither SSB nor DSB (Figure 4a and b, Supplementary Figure S5 lanes 7–12). Treatment of samples with Fpg + Endo III and analysis by neutral and glyoxal gel showed that PBSA photosensitized oxidized bases only at a concentration of 4 mM (Figure 4a and b, Supplementary Figure S5 lanes 1–6). When damaged bases were investigated by LMPCR, we observed that, as previously described, photosensitized bases were guanines (Figure 4c). As observed *in cellulo* after UV-B irradiation, the photosensitizing effect of PBSA was only present at concentrations superior to 0.5 mM, underlying the protective effect of a cellular context.

These data show that PBSA has a protective effect on CPD formation *in cellulo* as well. However, in the context of UV-A and UV-B irradiations, PBSA photosensitizes the formation of oxidized guanines. Markedly, the band intensities of guanine doublets show preferential oxidation of their 5′ guanine (Figures 3f and 4c), which indicates that, *in cellulo*, only type I photosensitization operates (Hiraku *et al.*, 2007). Finally, PBSA did not photosensitize SSB or DSB *in cellulo*, as opposed to *in vitro,* in which it generates DNA strand breaks after both UV-A and UV-B irradiations.

DISCUSSION

PBSA is a water-soluble sunscreen agent widely used in cosmetic formulations as it absorbs strongly at UV-B wavelengths. The maximal PBSA concentration approved by the Food and Drug Administration is 148 mM; usually concentrations ranging between 74 and 148 mM are found in commercial sunscreen preparations. An earlier study established that photoexcited PBSA could oxidize guanines in isolated DNA even at concentrations as low as 10 μM (Stevenson and Davies, 1999). Owing to its potential phototoxic effect, it is of great interest to determine whether PBSA can photosensitize the formation of DNA damage *in cellulo* on UV exposure. To this end, we have irradiated

isolated DNA and human normal primary fibroblasts with UV-A or UV-B in the presence of low concentrations of PBSA. We monitored DNA damage formation by converting modified bases to DNA SSB. CPDs were measured using the combined action of T4 endo V and photolyase, whereas oxidized purines and pyrimidines were measured using the combined action of Fpg and Endo III. Subsequently, the distribution of DNA fragment lengths was analyzed through neutral- or glyoxal-agarose gel electrophoresis. This combined approach of gel techniques makes it possible to monitor for DSBs (neutral gel) or both DSBs and SSBs (glyoxal gel). Moreover, neutral and glyoxal gels are particularly suited for DNA damage measurement, as they allow DNA molecules to be fractionated according to their size while retaining alkali-labile sites intact and thereby avoiding false signals (Drouin *et al.*, 1996a). Finally, LMPCR was used to determine which DNA bases were damaged in the presence of photo-activated PBSA.

We clearly observed that PBSA protected from CPD formation *in vitro*. However, in parallel to this protection, PBSA was capable of photosensitizing the formation of oxidized guanines and DNA strand breaks after UV-A and UV-B irradiation. Its ability to promote UVB-induced photosensitization of guanine bases in oligodeoxyribonucleotides has been noted previously (Stevenson and Davies, 1999). As expected, PBSA also protected from CPD formation *in cellulo*. However, its action as a photosensitizer differed somewhat from that observed *in vitro*. Indeed, although it also photosensitized the formation of oxidized guanines after UV-A or UV-B, it did not induce either SSB or DSB. Finally, the combined use of Fpg + Endo III revealed that PBSA did not induce the formation of measurable oxidized pyrimidines either *in vitro* or *in cellulo*.

From a mechanistic point of view, it has been shown that PBSA may function as a photosensitizer through both type I and type II photosensitization mechanisms with the guanine moieties of oligodeoxynucleotides (for review of photosensitization mechanisms, see reference (Cadet *et al.*, 2009)). As guanines in guanine doublets were oxidized identically whether they were in 5′ or 3′ position *in vitro* after UV-A and UV-B, PBSA most likely photosensitized these damages at least through type II mechanism (Hiraku *et al.*, 2007). However, we could not rule out that type I mechanism also operated. As DNA strand breaks were only observed at guanine, we propose that they were the consequence of overoxidation of oxidized guanines (Burrows and Muller, 1998; Cadet *et al.*, 2008). This mechanism might be favored *in vitro,* in which the extent of base damage is much higher than that in cells, thus allowing possible occurrence of secondary reactions of initially generated 8-oxo-7,8-dihydroguanine (8-oxoGua), a highly photosensitive target.

In cells, if PBSA is located in the vicinity of DNA, one-electron oxidation (type I mechanism) of guanines might occur and gives rise to 8-oxoGua and 2,6-diamino-4-hydroxy-5-formamidopyrimidine without inducing DNA strand breaks. Moreover, the superoxide radical, which may be produced by reaction of O_2 with PBSA radical anions, arising from electron transfer from a suitable donor to the triplet-excited photosensitizer, may in turn yield highly reactive •OH through a Haber–Weiss reaction. In addition to 8-oxoGua, the latter free radical is able to generate 2,6-diamino-4-hydroxy-5-formamidopyrimidine and several oxidation products of pyrimidine bases, as well as strand breaks, as the result of hydrogen abstraction from the 2-deoxyribose moiety. A second likely photoreaction of UV-A-excited PBSA is the generation of singlet oxygen (type II mechanism), a slightly diffusible reactive species (Kuimova *et al.*, 2009), the action of which leads to the exclusive formation of 8-oxoGua in cellular DNA. *In cellulo,* the only base damage that we observed comprised oxidized guanines. As no strand breaks were observed and guanines doublets showed preferential oxidation of the 5′ guanines, we propose that type I mechanism is involved.

To explain the photosensitizing effect of PBSA observed *in cellulo* after UV-A irradiation, we propose three non-exclusive possibilities. First, the photosensitization of oxidized bases after UV-A might be explained by weak absorption of PBSA at 320–330 nm in the UV-A wavelength range. Indeed, although the UV-A source used in these experiments emits maximally at 350 nm, trace amounts of radiation are also emitted at shorter wavelengths that overlap the PBSA absorption spectrum. Second, we can also envision that PBSA itself becomes a UV-A photoreactive molecule during specific circumstances. Indeed, Serpone *et al.* (2002) showed that PBSA diluted in water is degraded and shows absorption abilities of UV-A wavelength after simulated sunlight irradiation. Finally, we can speculate whether the oxidative cellular stress observed after UV-A irradiation might lead to PBSA oxidation products exhibiting DNA photosensitization properties.

In conclusion, our work shows that, *in cellulo,* PBSA provides efficient protection against CPD formation during UV-B exposure. Nevertheless, under UV-A or UV-B irradiation, PBSA promoted DNA damage, as it indirectly photosensitized the formation of oxidized guanines. PBSA can thus function as a double-edged sword toward DNA even at 4 mM, which is a very low concentration as compared with the 147 mM authorized by the Food and Drug Administration. These data are of major importance, as several studies have clearly shown that sunscreen agents are able to penetrate the skin and can be detected in human breast milk and urine (Hayden *et al.*, 1997; Hanson *et al.*, 2006). The usefulness of PBSA in sunscreen applications is thus questionable because of its photo-oxidizing properties. Nevertheless, it has been recently shown that complexation of PBSA with cyclodextrin inhibits the formation of free radicals generated by PBSA during exposure to simulated sunlight (Scalia *et al.*, 2004). It would be interesting to determine whether this sequestered form of PBSA shares the same photosensitizing properties that we have observed with noncomplexed PBSA. Finally, this study emphasizes that interactions between cell components, chemical molecules, and UV light can involve very complex mechanisms. *In cellulo* experiments are essential to fully assess the phototoxicity of chemical compounds, especially when they are used in connection with human health.

MATERIALS AND METHODS

Equipment, chemicals, and enzymes

UV-A and UV-B doses were measured using a UVX Digital Radiometer and its appropriate probes (UVP Inc., Upland, CA). The UV-B source consisted of two fluorescent tubes, FS20T12/UVB/BP (Philips, Franklin Square Drive, NJ), delivering a fluence rate of 3.39 J m^{-2} $second^{-1}$, which was filtered through a 0.015-inch sheet of clear cellulose acetate Kodacel TA-407 (Eastman Kodak, Rochester, NY) to eliminate wavelengths below 290 nm. The UV-A source was composed of two fluorescent tubes, F15T8BLB 15 W Black light Blue (Sankyo Denki, Tokyo, Japan), delivering a fluence rate of 31 J m^{-2} $second^{-1}$.

PBSA was obtained from Sigma (Sigma, St Louis, MO). T4 endonuclease V and DNA photolyase (*Escherichia. coli*) were kindly provided by Dr RS Lloyd (University of Texas Medical Branch, Galveston, TX) and Dr A Sancar (University of North Carolina, Chapel Hill, NC), respectively. Endo III and Fpg were kindly provided by Dr S Boiteux (Commissariat à l'Energie Atomique, Fontenay aux Roses, France). LMPCR primers were purchased from IDT (Coralville, IA). *Taq* DNA polymerase and T4 DNA ligase were purchased from Roche Applied Science (Laval, QC, Canada). *Pfu* exo^- DNA polymerase was purchased from Stratagene (La Jolla, CA). All primer extensions and PCR amplifications were carried out on a TGradient Thermocycler (Biometra, Göttingen, Germany).

All solutions used for the extraction, enzymatic digestion, and irradiation of DNA were prepared in ''nanopure'' H_2O.

Dermal fibroblast culture

The study was conducted according to the Declaration of Helsinki Principles. All fibroblasts used in this project were purchased from Clonetech Laboratories Inc. (Palo Alto, CA). Fibroblasts (2×10^6) were seeded in 75 cm^2 flasks (Falcon, Becton–Dickinson, Cockeysville, MD) and grown in Dulbecco's Modified Eagle's Medium (Invitrogen Life Technologies, Carlsbad, CA) containing 10% fetal calf serum (Invitrogen Life Technologies, Carlsbad, CA), 100 U ml^{-1} penicillin G, 25 μg ml^{-1} streptomycin, and 0.5 μg ml^{-1} fungizone. For UV irradiations, fibroblasts were grown in Petri dishes and used once the culture had reached 90% confluence.

UV irradiations

Fibroblasts for *in cellulo* UV exposure, as well as purified DNA for *in vitro* exposure, were preincubated for 30 minutes with PBSA and then irradiated on ice. Purified DNA irradiations were carried out at a concentration of 65 μg ml^{-1} in irradiation buffer consisting of 0–4 mM PBSA, 150 mM NaCl, 10 mM KCl, 1 mM EDTA, and 10 mM Tris-HCl (pH 7.4). For fibroblasts grown as monolayers in 150 mm Petri dishes, cells were washed once with 1 × PBS (137 mM NaCl, 2.7 mM KCl, 100 mM Na_2HPO_4, and 2 mM KH_2PO_4); thereafter, irradiations were carried out in 10 ml of 1 × PBS containing 0–4 mM PBSA.

DNA extraction

Human primary fibroblast DNA was extracted as previously described (Drouin *et al.*, 2009).

Digestion of DNA with damage-specific enzymes

CPDs were digested as follows: 10 μg of each DNA sample was incubated for 1 h at 37°C in 100 μl of T4 endo V reaction mixture (50 mM Tris-HCl (pH 7.6), 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 0.1 mgml^{-1} BSA containing T4 endonuclease V). Thereafter, 10 μl of photolyase reaction mix (50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 1 mg ml^{-1} BSA containing DNA photolyase) was added. Photoactivation of the photolyase was achieved by placing opened tubes covered with a plastic film 2–3 cm from a UV-A black light for 1 h.

Digestion of oxidized bases was performed by incubating 10 μg DNA samples for 1 h at 37°C in 100 μl of Fpg + Endo III reaction mixture (40 mM 4-(2-hydroxyethyl)-1piperazineethanesulfonic acid, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg ml^{-1} BSA containing 2.1 ng μl^{-1} of Endo III and 2 ng μl^{-1} of Fpg, pH 8). Reaction was then stopped by adding 300 μl of stop mixture (0.6% SDS).

At the end of both digestions, DNA was purified using phenol–chloroform extractions.

Neutral-agarose gel electrophoresis

Samples containing 0.2 μg of DNA in 10 μl water were mixed with 2 μl of gel-loading buffer 6 × (0.25% bromophenol blue, 0.25% xylene cyanol FF and 30% glycerol). Samples were then loaded onto a 0.8% agarose gel (agarose A, Laboratoire Mat, Beauport, QC, Canada) and electrophoresis was performed at a constant voltage of 60 V in 1 × tris-acetate EDTA buffer. DNA was stained by incubating gels for 3 hours in an aqueous solution of 1 × SYBR gold (Molecular Probes, Eugene, OR). DNA strand break frequency was determined using the method described by Willis *et al.* (1988; Drouin *et al.*, 1996a). Gel quantifications were performed using ImageQuant 5.0 (Molecular Dynamics, Sunnyvale, CA).

Glyoxal-agarose gel electrophoresis

Samples containing 0.2 μg of DNA were incubated for 1 hour at 50°C in 20 μl of glyoxal denaturing mixture (1.05 M glyoxal, 50% DMSO, and 10 mM sodium phosphate). After denaturation, samples were placed on ice and 3.8 μl of glyoxal-loading buffer was added (50% glycerol, 10 mM sodium phosphate pH 7, 0.25% bromophenol blue, and 0.25% xylene cyanol FF). Samples were then loaded onto a 0.8% agarose gel (agarose low Electro-Endo-Osmosis, Boehringer Mannheim GmbH, Mannheim, Germany). Electrophoresis was performed at a constant voltage of 60 V in 10 mM sodium phosphate buffer. DNA was stained by incubating gels for 3 hours in an aqueous solution of 1 × SYBR gold (Molecular Probes). DNA strand break frequency was determined using the method described by Willis *et al.* (1988; Drouin *et al.*, 1996a). Gel quantifications were performed using ImageQuant 5.0 (Molecular Dynamics, Sunnyvale, CA).

Ligation-mediated PCR

The LMPCR procedure was conducted as previously published (Angers *et al.*, 2001; Drouin *et al.*, 2009). The distribution of photosensitized oxidized bases was determined along the nontranscribed strand of the *p21* gene. The LMPCR primers used to study the distribution of oxidized bases at the *p21* locus have been described (Millau *et al.*, 2009). The primer set used here was 1.1 (5′-GGGTCTGCTCTGTGTCC-3′), 1.2 (5′-CCCACCCCTACCTGGGC TCCCATCC-3′), and 1.3 (5′-CCACAGCAGAGGAGAAAGAAGCC-3′), and the corresponding nucleotide positions localized at −1209 bp from the transcription start site of *p21*.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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