



Melanin Photosensitization and the Effect of Visible Light on Epithelial Cells

Orlando Chiarelli-Neto¹, Alan Silva Ferreira¹, Waleska Kerllen Martins¹, Christiane Pavani¹, Divinomar Severino¹, Fernanda Faião-Flores², Silvyta Stuchi Maria-Engler², Eduardo Aliprandini³, Glauca R Martinez³, Paolo Di Mascio¹, Marisa H. G. Medeiros¹, Maurício S. Baptista^{1*}

1 Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, São Paulo, Brazil, **2** Departamento de Análises Clínicas, Faculdade de Ciências Farmacêuticas-USP, São Paulo, Brazil, **3** Departamento de Bioquímica e Biologia Molecular, Setor de Ciências Biológicas, Universidade Federal do Paraná, Curitiba, Brazil

Abstract

Protecting human skin from sun exposure is a complex issue that involves unclear aspects of the interaction between light and tissue. A persistent misconception is that visible light is safe for the skin, although several lines of evidence suggest otherwise. Here, we show that visible light can damage melanocytes through melanin photosensitization and singlet oxygen (¹O₂) generation, thus decreasing cell viability, increasing membrane permeability, and causing both DNA photo-oxidation and necro-apoptotic cell death. UVA (355 nm) and visible (532 nm) light photosensitize ¹O₂ with similar yields, and pheomelanin is more efficient than eumelanin at generating ¹O₂ and resisting photobleaching. Although melanin can protect against the cellular damage induced by UVB, exposure to visible light leads to pre-mutagenic DNA lesions (i.e., Fpg- and Endo III-sensitive modifications); these DNA lesions may be mutagenic and may cause photoaging, as well as other health problems, such as skin cancer.

Citation: Chiarelli-Neto O, Ferreira AS, Martins WK, Pavani C, Severino D, et al. (2014) Melanin Photosensitization and the Effect of Visible Light on Epithelial Cells. PLoS ONE 9(11): e113266. doi:10.1371/journal.pone.0113266

Editor: John R. Battista, Louisiana State University and A & M College, United States of America

Received: August 1, 2014; **Accepted:** October 6, 2014; **Published:** November 18, 2014

Copyright: © 2014 Chiarelli-Neto et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper and its Supporting Information files.

Funding: FAPESP (12/50680-5; 13/07937-8), CNPq, INCT Redoxoma (FAPESP/CNPq/CAPES; Proc. 573530/2008-4) and FARMA Service Bioextract are acknowledged for providing funds for this research. OCN acknowledges FAPESP for his PhD fellowship (2010/08796-0). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* Email: baptista@iq.usp.br

Introduction

Humans and other animals produce melanin mainly for protection against exposure to ultraviolet B (UVB) radiation [1]. In contrast to UVB radiation, which is directly absorbed by DNA, UVA radiation acts essentially through photosensitization, which generates a triplet species, ¹O₂, and subsequently generates other radical species that can damage both DNA and other epithelial cell biomolecules [2–4]. UVA penetrates deeper in the dermis than does UVB, and it is the major UV source responsible for skin photoaging and the development of several types of skin cancer [5].

However, the visible portion of the spectra has garnered much less attention, despite several scientific reports that have described the effect of visible and IR irradiation on the skin [6,7]. Kielbassa and co-workers [8] and Kvam and Tyrrel [9] showed that irradiating Chinese hamster cells and dermal fibroblasts, respectively, with UVA and visible light induced oxidative damage in DNA. More recent studies showed that visible light disturbs the epidermal barrier, and this disturbance induces pigmentation and inflammatory responses [10,11]. However, a great deal of controversy remains concerning the effect of visible light on the skin, most likely because of the lack of a mechanism that explains the observed effects [12].

It has been shown that, in addition to UV [13], visible light also induces pigmentation in certain skin types. Mahmoud and co-workers [11] showed that visible light induces skin darkening in people with skin types IV and V but not in individuals with type II skin. The darkening induced by visible light depended on the pre-irradiation melanin content of the skin, suggesting that melanin may directly damage skin cells upon exposure to visible light.

The literature describes both protective and damaging roles for melanin [14,15]. Two independent studies using *Xiphophorus*, which is a fish that is highly susceptible to melanoma, showed that the action spectra for both melanoma induction [16] and the photo-induced generation of reactive species [17] extend to visible wavelengths and that the shapes of the action spectra correspond to the shape of the melanin absorption spectrum. Despite the lack of a mechanistic explanation for the observations, these articles highlight the importance of understanding the role of the excited species that are generated after melanin is excited by visible light. In an earlier publication, we showed that melanin can act as a photosensitizer, leading to ¹O₂ generation after excitation with visible light [18]. Singlet oxygen can react with proteins, nucleic acids and membranes [19] (Figure S1); consequently, melanin photosensitization is likely involved in the phototoxicity of visible light, which is the main hypothesis that we aim to demonstrate herein.

Methods

Reagents

All solvents were spectroscopic grade. Water was distilled from an all-glass apparatus and further purified via a Millipore Milli-Q system. D₂O (99%), tyrosine (Tyr), ammonium chloride (NH₄Cl), the enzymes Fpg and Endo III, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), KCl, Na₂EDTA, HEPES and BSA from Sigma-Aldrich (either USA or Germany) were used as received. All other materials were the best analytical grade available. NaOD was prepared using three cycles of dissolution and evaporation of the initial NaOH solid (1 g) with D₂O (10 g). The naphthalene derivative endoperoxide (DHPNO₂) was synthesized by photosensitizing the precursor *N, N'*-di(2,3-dihydroxypropyl)-1,4-naphthalenedipropanamide (DHPN) with methylene blue as described by C. Pierlot et al [20]. The endoperoxide concentration was determined for each aliquot after the final filtration. The solutions were obtained at concentrations between 170 to 190 mmol/L with a purity of approximately 84%. This purity value refers to the amount of DHPNO₂ obtained relative to the total amount of (DHPNO₂+DHPN).

Eumelanin and pheomelanin were synthesized as described by Hayward et al with modifications [21]. Eumelanin was prepared from L-tyrosine (2.5 mg/mL) in pH 7.4 phosphate buffer (50 mM) and mushroom tyrosinase (150 U/mL) in bovine serum albumin (BSA) solution (5 mg/mL). Pheomelanin was synthesized from L-dopa (0.5 mg/mL) and L-cysteine (1.5 mg/mL) in pH 7.4 phosphate buffer (50 mM) and mushroom tyrosinase (100 U/mL). The reactions were performed at room temperature with stirring for 24 h. We also used eumelanin and pheomelanin samples that were kindly provided by Dr. S. Ito [22]. The samples from our lab and those from Dr. Ito's lab behaved identically with regard to ¹O₂ generation.

Equipment

The visible and UV light irradiator (Novatecnica, Brazil) included temperature and humidity sensors. Both variables were maintained during the experiments. The irradiation (mW/cm²) was measured at eight different areas in this irradiator using a dosimeter (VLX-3W, France). The irradiances of the light source were 3.0 mW.cm⁻² and 3.3 mW.cm⁻² in the UVB and visible regions, respectively. For UVB, 25 min of irradiation provided 4.5 J.cm⁻². For the visible region, 30 min of irradiation provided 6 J.cm⁻², 180 min of irradiation provided 36 J.cm⁻², and 360 min of irradiation provided 72 J.cm⁻².

Cell absorption/emission was quantified using a plate reader (Tecan Infinite 200M USA). The ¹O₂ measurements were performed in a specially designed instrument [18,19,23] consisting of a Surelite III laser (355 nm and 532 nm, 5-ns pulses, 10 pulses/s, 1 mJ/pulse; Continuum Lasers), cuvette holder, silicon filter, monochromator, liquid-nitrogen-cooled near infrared photomultiplier tube (NIR-PMT R5509) from Hamamatsu (Hamamatsu Co., Bridgewater, NJ, USA) and a fast multiscaler analyzer card with a resolution of 5 ns/channel (MSA-300; Becker & Hickl, Berlin, Germany). The signal was acquired either from a cell cuvette or directly under a fluorescence microscope (Nikon Eclipse Ti, USA). Fluorescence/transmission microscopy images were acquired from an Axiovert 200 microscope or an LSM 510 laser confocal microscope (Zeiss, Germany). The comet assay images were obtained using fluorescence microscopy (Olympus BH-2, USA). ImageJ Launcher was used for the confocal image analyses (National Institutes of Health, Bethesda).

Cell culture

Several cell lines, which are available commercially, were received as gift: B16-F10 [24]; HaCaT [25]; J774 [26] and SK-mel 28 [27]. B16-F10, HaCaT and HeLa cells were cultivated in Dulbecco's Eagle (DMEM) culture medium (Sigma-Aldrich). J774 and SK-mel cells were cultivated in RPMI 1640 culture medium (Sigma-Aldrich). Both media were supplemented with 10% SFB (Gibco/BRL Life Technologies), 4 mM L-glutamine (Sigma USA), 100 U/mL penicillin (Sigma USA) and 100 mg/mL of streptomycin (Nissui Seiyaku) and incubated at 5% CO₂ and 37°C. Primary skin cell cultures (melanocytes) were obtained from the foreskins of University Hospital (Hospital Universitário – HU-USP) patients [28]. The project was reviewed and approved by the Research Ethics Committee of the University Hospital (Av. Prof. Lineu Prestes, 2.565-Cidade Universitária-CEP 05508-000; + (5511)30919200, São Paulo, Brazil) (protocol# 943/09). The experiments were performed with each subject's understanding and written consent, and the study methodologies conformed to the standards set by the Declaration of Helsinki. The melanocytes were maintained in 254CF medium (SKU# M-500-254CF; Cascade Biologics, USA) with human melanocyte growth supplement (HMGS – SKU# S-002-5; Cascade Biologics, USA).

Melanogenesis, irradiation and viability

The cells were plated (2×10^4 cells.mL⁻¹) and, after 24 h, were treated with 0.5 mM Tyr (Sigma-Aldrich, Germany) and 10 mM NH₄Cl (Labsynth, Brazil) for 48 h. This protocol increases the melanin production of melanocytes, and the resulting melanocytes and are referred to as M++++ herein; the control cells are referred to as CT. The cells were irradiated in PBS (8 g/L NaCl, 0.20 g/L KCl, 1.15 g/L Na₂HPO₄, and 0.2 g/L KH₂PO₄). We applied 4.5 J.cm⁻² of UVB irradiation and 36 J.cm⁻² and 72 J.cm⁻² of visible irradiation. The cell density was evaluated using acridine orange fluorescence (excitation 488 nm, emission 515 nm). The cell viability was evaluated using MTT colorimetric and crystal violet assays [29]. Damage to the cytoplasmic membrane was quantified using propidium iodide incorporation. Apoptotic cell death was characterized using caspase-3 activation (Cell Signaling Technology, USA).

B16-F10 cell viability after 24 h of a DHPNO₂ treatment

The B16-F10 cells (CT and M++++) were treated with a solution containing RPMI and 10 mM DHPNO₂ in the absence of serum for 2 h. In control assays, the cells were treated with a solution containing RPMI and 10 mM DHPN (decomposition product of DHPNO₂) in the absence of serum for the same time period. After this period, the cell medium was changed to a normal culture medium (RPMI 1640 (Cultilab) supplemented with fetal calf serum (7.5%) from Gibco and with sodium bicarbonate (0.8 mM), HEPES (20 mM), and gentamicin (50 ng/mL) from Sigma-Aldrich. Cell viability was determined using an MTT assay 24 h after the DHPNO₂ treatment.

Melanin quantification

The melanin content was quantified as previously described [30]. The B16-F10 cells were seeded in 96-well plates (1×10^4 cells.mL⁻¹), and after 24 h, they were treated with 0.5 mM Tyr (Sigma-Aldrich Germany) and 10 mM NH₄Cl (Labsynth Brazil) for 48 h. After incubation, a portion of the cells was centrifuged and suspended in 1 M NaOH (Labsynth Brazil). The other portion was maintained in PBS (8 g/L NaCl, 0.20 g/L KCl, 1.15 g/L Na₂HPO₄, and 0.2 g/L KH₂PO₄) for protein quantification. Both aliquots were lysed in a Branson Sonifier 450 (USA)

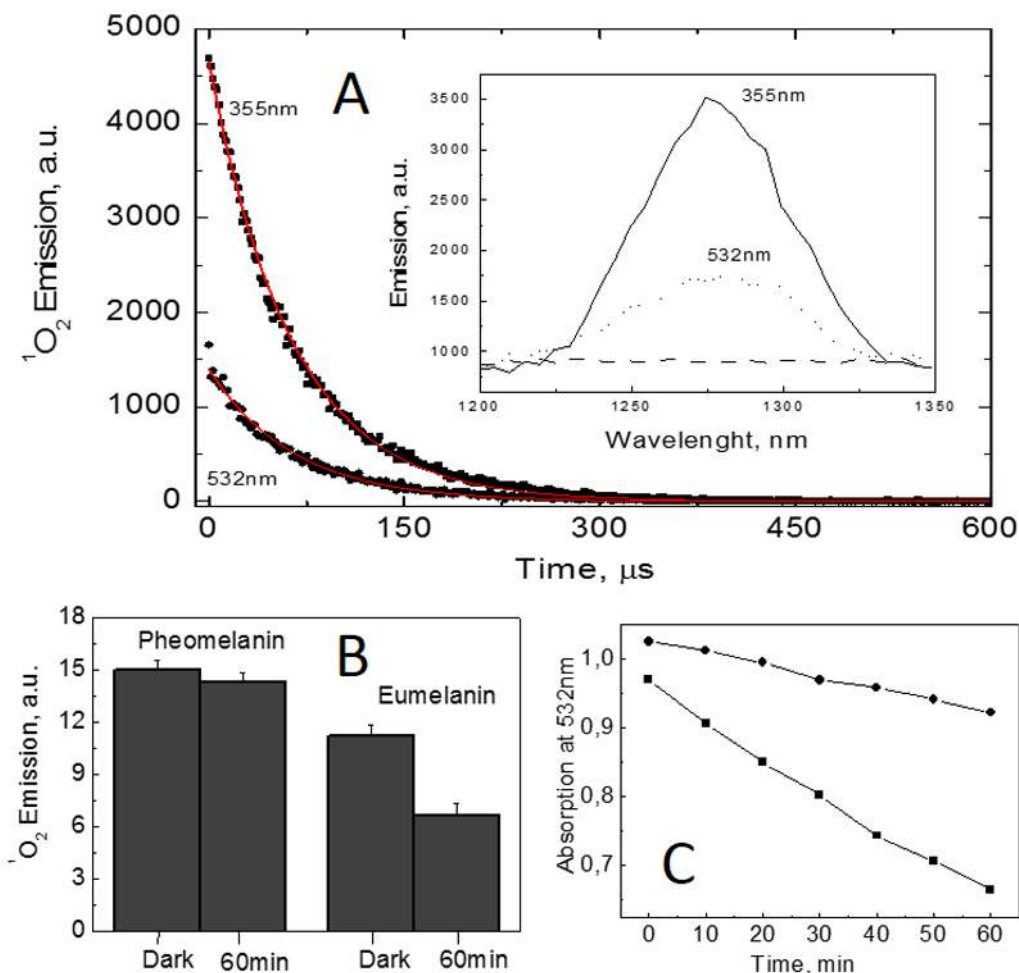


Figure 1. $^1\text{O}_2$ generation from melanin. (A) $^1\text{O}_2$ decay from a eumelanin solution ($0.04 \text{ g}\cdot\text{L}^{-1}$) in acetonitrile, $\text{pD}=10$ with excitation at either 355 or 532 nm. The $^1\text{O}_2$ decay lifetimes were the same under both conditions ($\sim 17 \mu\text{s}$, which is the lifetime expected for a mixture of acetonitrile and water) [18]. The insert shows the near infrared emission spectra after exciting a eumelanin solution at 532 nm and 355 nm in the absence of azide (continuous lines) and in the presence of 3 mM azide (dashed flat line); for the excitation pulses at both 355 nm and 532 nm, we used the following parameters: 5 ns, 10 pulses/s, and 1 mJ/pulse. (B) An integral of the emission spectra from pheomelanin ($\text{OD}=1.02$) and eumelanin ($\text{OD}=0.97$) solutions in acetonitrile immediately following dissolution (dark) and after 60 min of irradiation with visible light (light dose of $12 \text{ J}\cdot\text{cm}^{-2}$). (C) Absorption as a function of irradiation time for pheomelanin (\bullet) and eumelanin (\blacksquare) solutions in acetonitrile. doi:10.1371/journal.pone.0113266.g001

at 20 W for 30 sec. The melanin was quantified by measuring the absorption at 470 nm in a Tecan Infinite 200M plate reader using a standard curve for commercial melanin (Sigma Aldrich Germany) [30]. The total protein content was determined using the Bradford method [31]. Melanin is expressed as μg of melanin/mg of protein.

Comet assay

Comet assays were performed according to optimized protocols [32,33]. B16-F10 cells were plated at $1 \times 10^5 \text{ cells}\cdot\text{mL}^{-1}$ in culture medium. Twenty-four hours after seeding, the cells were treated with 0.5 mM Tyr (Sigma-Aldrich, Germany) and 10 mM NH_4Cl (Labsynth, Brazil) for 48 h. After incubation, the cells were irradiated in PBS (8 g/L NaCl, 0.20 g/L KCl, 1.15 g/L Na_2HPO_4 , and 0.2 g/L KH_2PO_4) using $36 \text{ J}\cdot\text{cm}^{-2}$ or $6 \text{ J}\cdot\text{cm}^{-2}$ of visible light. We mixed 30 μl of a 1×10^5 cell suspension with 100 μl of agarose (Sigma USA) (0.5% concentration in PBS) and distributed the mixture on slides that were pre-coated with agarose (Sigma USA) (1.5% concentration in PBS) and incubated on ice.

After solidifying, the cells were lysed in the dark using a high-salt alkaline buffer (0.5 M NaCl, 0.1 M EDTA, 0.01 M Tris, and 1% Triton X-100, pH 10). For samples irradiated with $36 \text{ J}\cdot\text{cm}^{-2}$ or $6 \text{ J}\cdot\text{cm}^{-2}$, the slides were placed in electrophoresis buffer (0.3 M NaOH and 1 mM EDTA, pH 13, cooled in a refrigerator) in the dark for 30 min. Electrophoresis was performed in a cold-storage room, in the dark, using a power supply (ESP 301; GE USA) with the same buffer for 30 min at 25 V. After electrophoresis, the slides were neutralized using 0.4 M Tris at pH 7.5 and fixed in ethanol. In the protocol used to evaluate the direct DNA damage from melanin photosensitization, the cells were treated with $6 \text{ J}\cdot\text{cm}^{-2}$ of visible light, and before electrophoresis, the slides were treated with 0.2 U of Fpg or Endo III enzymes (Sigma-Aldrich, USA) in buffer (0.1 M KCl, 0.5 mM Na_2EDTA , 40 mM HEPES and 0.2 mg/mL BSA at pH 8.0) for 30 minutes at 37°C . Subsequently, the DNA was stained with ethidium bromide ($10 \mu\text{g}\cdot\text{mL}^{-1}$), excited at 515 nm and observed using a fluorescence microscope (Olympus BH-2, USA) [34].

¹O₂ generation

The B16-F10 cells were seeded in six-well plates (2×10^5 cells.mL⁻¹), and after 24 h, they were treated with 0.5 mM Tyr (Sigma-Aldrich, Germany) and 10 mM NH₄Cl (Labsynth, Brazil) for 48 h. The cells were washed in PBS (8 g/L NaCl, 0.20 g/L KCl, 1.15 g/L Na₂HPO₄, and 0.2 g/L KH₂PO₄), removed from the plates using a cell scraper and resuspended in D₂O saline solution. We obtained ¹O₂ emission spectra by measuring the emission intensities from 1180 to 1360 nm with 1- to 5-nm steps using the equipment described above.

Statistical analysis

The experiments were performed with least as three independent repetitions. The statistical analyses were assessed using Student's t test and Microcal Origin software (version 7.0); $P < 0.05$ was considered statistically significant.

Results

The excitation of melanin with either UVA (355 nm) or visible (532 nm) light generated the characteristic ¹O₂ NIR emission spectra with a maximum centered at 1270 nm, which is a fingerprint of the O₂ ($a^1\Delta_g \rightarrow O_2(X^3\Sigma_g^-)$) transition (Fig. 1). Sodium azide suppression confirmed this assignment (Fig. 1B, insert) [18,19]. The emission intensity was stronger after melanin was excited by UVA than after excitation by visible light, but this difference is due to the greater melanin absorption at 355 compared with that at 532 nm; thus, the ¹O₂ generation efficiency does not vary depending on source of irradiation (UVA or visible).

Furthermore, the ¹O₂ emission from pheomelanin was 30% more intense than that from eumelanin (Fig. 1B), and visible light irradiation substantially decreased the absorption (Fig. 1C) and the generation of ¹O₂ by eumelanin (Fig. 1B); in contrast, the decrease (~7%) in ¹O₂ generation by pheomelanin was substantially smaller (Fig. 1B). The chemical reaction underlying eumelanin photobleaching is the addition of ¹O₂ to the double bond at the C3 of the indole group with the consequent hydroperoxide formation [18]. This type of photoproduct was not detected from the pheomelanin photolysis.

To understand the potential effects of melanin photosensitization on epithelial cells, we compared the UVB and visible light photosensitivity using cell lines that express different amounts of melanin (Fig. 2). The irradiation doses were selected to mimic the exposure of an individual to ~10 minutes of a sunny day in Brazil. Notably, the cells expressing more melanin had a higher survival rate after they were challenged with UVB, which is consistent with previous results [30]. However, the darker cells suffered from high phototoxicity from visible irradiation (Fig. 2), providing strong evidence that the phototoxicity from visible irradiation is related to the amount of melanin.

We then tested the visible light toxicity in two melanocompetent cell lines (B16-F10 and human Caucasian melanocytes) under two different melanin production regimens (Fig. 3; i.e., basal level or control (CT) and induced melanogenesis (M++++)) [30]. Melanocytes in a culture clearly differ from skin melanocytes; thus, this system is a well-known, good experimental model for testing the cellular response to environmental challenges [35].

UVB exposure reduced the viability by 40% in B16-F10 CT (left), while pigmented cells (M++++, right) showed only a ~9% reduction (Fig. 3). Therefore, the higher melanin content protected the pigmented cells from UVB damage, which is consistent with previous data from the literature [30,36,37]. The effect of visible light was opposite to the effect observed for UVB irradiation. Furthermore, upon irradiation with visible light (36 J.cm^{-2}), the

control cells for both the Caucasian melanocytes (H36) and the B16-F10 (M36) cells (left side, Fig. 3B) only showed a ~5% decrease in cell viability. At 72 J.cm^{-2} , the viability decrease was also small (i.e., 15%; M72, left side, Fig. 3B). However, when the cells were pigmented and treated with 36 or 72 J.cm^{-2} of visible light, both cell lines exhibited substantial decreases in viability (50% for H36, 25% for M36 and 40% for M72), which clearly demonstrates that the presence of melanin increases visible light phototoxicity. Moreover, the comet assay showed that the level of DNA fragmentation was higher in the pigmented cells than in the CT cells (Fig. 3B-images), which is consistent with increased visible light phototoxicity for the increased level of intracellular melanin. The mechanism of cell death was mainly necro-apoptosis; substantial levels of propidium iodide were incorporated (cytoplasmic membrane damage, Fig. 3C), and caspase 3 was activated (Fig. 3D).

To correlate the visible light phototoxicity with the melanin and ¹O₂ contents, we quantified the amount of ¹O₂ generated in cells by measuring the near-infrared emission spectrum (1270 nm) after excitation with visible light (532 nm) [18] (Fig. 4A). The control cells only exhibited background signals (Fig. 4A, insert). However, the pigmented B16-F10 cells (M++++) showed the characteristic ¹O₂ spectra with a maximum intensity at approximately 1270 nm (the darker line in the Fig. 4A insert). Thus, the higher intracellular melanin content in the pigmented cells corresponded to more ¹O₂ generation and higher phototoxicity in response to visible irradiation compared with those for the control cells. To establish a definitive relationship between the ¹O₂ level and the cell toxicity, we also generated this species in the intracellular environment using a clean ¹O₂ source (i.e., thermal decomposition of DHPNO₂). As shown in Fig. 4B, the cells treated with DHPNO₂ exhibited a substantial decrease in viability (~50%) compared with the viability of both types of control (cells without treatment and cells challenged with DHPN), which demonstrates that ¹O₂ plays a role in the reduced cell viability (Fig. 4B). Furthermore, the generation of ¹O₂ slightly reduced the viability of CT cells (49.7%) compared with that of M++++ cells (53.3%). This difference was not statistically significant, possibly because melanin can suppress ¹O₂. As reported earlier, melanin can exert both types of effects, but in the presence of sufficient visible light illumination, melanin will stimulate the generation of ¹O₂ [14,18,38].

The excitation of melanin using visible light generates ¹O₂ and, consequently, the triplet species derived from melanin. Therefore, cellular damage can occur by both a type I mechanism (direct reaction between the triplet photosensitizer and biological targets, typically through an electron transfer reaction) and a type II mechanism (energy transfer reaction between the triplet photosensitizer and oxygen-forming ¹O₂) [19]. Depending on the severity of the damage, cell death will be the main outcome from visible light exposure. Another potential outcome, which is potentially more dangerous, is the generation of oxidative DNA products, which could lead to mutagenic compound accumulation, genomic instability and cancer [39].

To demonstrate the direct damage to nuclear DNA by the melanin photosensitization that occurs in response to visible light irradiation, we performed a comet assay under low-dose conditions (i.e., a light dose that does not measurably decrease the cell viability for both CT and M++++ cells (6 J.cm^{-2})). After irradiation, the cells were treated with endonuclease enzymes (Fpg and Endo III) that recognize specific types of oxidative damage in DNA (Fig. 5). Fpg recognizes 8-oxoguanine, 8-hydroxyguanine and formamidopyrimidine, and Endo III recognizes strand breaks, abasic sites and additional oxidative pyrimidine modifications [40]. For a control, the comet assay was repeated in the absence of endonucleases. As expected, under this mild condition, both the CT and pigmented cells in the

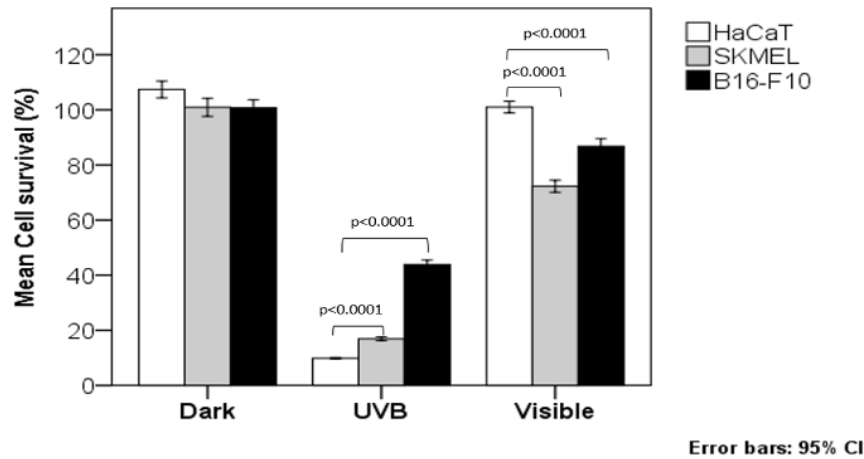


Figure 2. The effects of UVB and visible light on cell viability. The cell viability (MTT) of HaCaT, SK-mel and B16-F10 cells maintained in the dark (control) or irradiated with UVB (4.5 J.cm^{-2}) or visible (36 J.cm^{-2}) light. The color of the bars indicates the (qualitative) levels of melanin naturally produced by each cell line. Other cell lines, which are not melanocompetent (Hela, J774), were also tested and behaved similarly to the HaCaT cells (i.e., exhibited no phototoxicity from visible light at this dose (36 J.cm^{-2})). doi:10.1371/journal.pone.0113266.g002

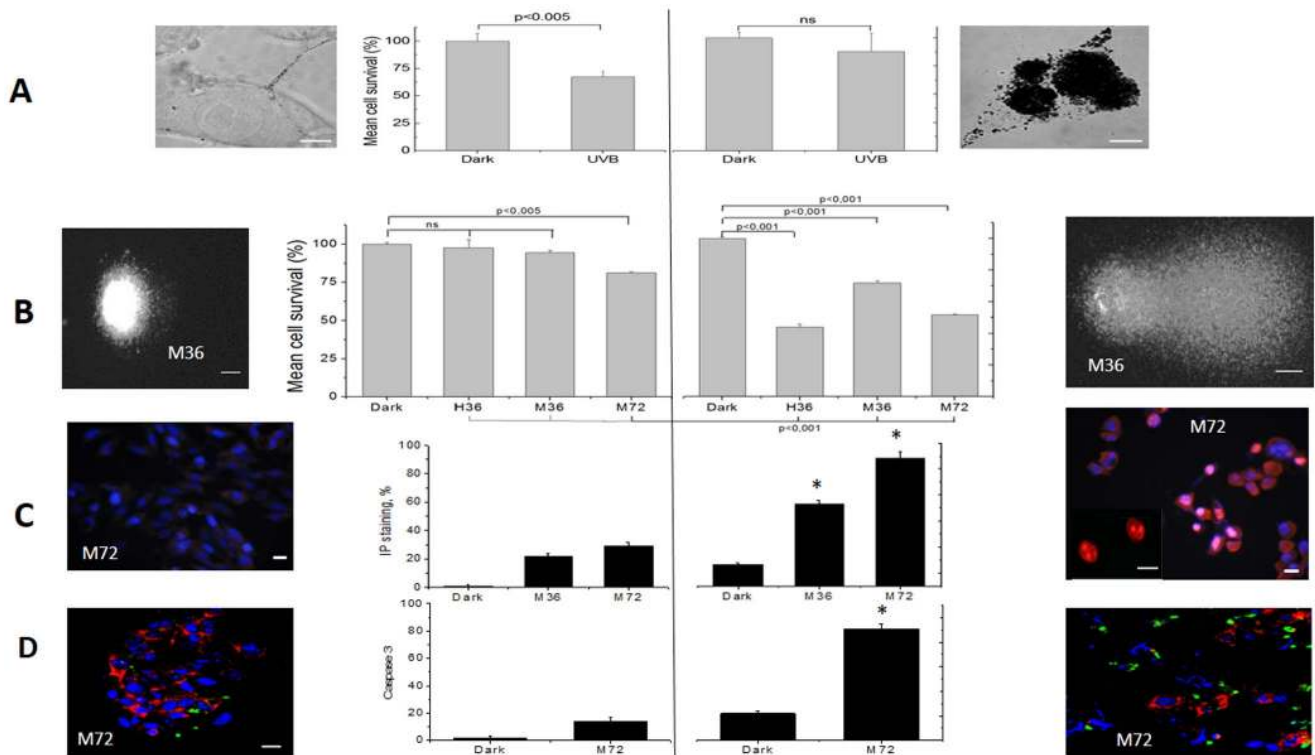


Figure 3. The effects of UVB and visible light in B16-F10 and human melanocyte cells. Left side: (no extra pigmentation); right side: cells subjected to the pigmentation protocol (M++++). (A) Viable cells (%) determined using the acridine orange fluorescence of B16-F10 cells that were maintained in the dark or with 4.5 J.cm^{-2} of UVB irradiation. The images are confocal optical microscopy images of the B16-F10 cells, CT (left) and M++++ (right). (B) The viable cells (%) were determined using the MTT colorimetric assay in the dark or with 36 J.cm^{-2} or 72 J.cm^{-2} of visible irradiation; murine B16-F10 cells are marked as either M36 or M72 depending on the light dose received. The human melanocytes (H36) only received a light dose of 36 J.cm^{-2} . The images on the sides show comet assays performed using the CT and M++++ B16-F10 cells, 180 min after irradiation with a light dose of 36 J.cm^{-2} . (C) Propidium iodide incorporation in B16-F10 CT and M++++ cells in the dark and after treatment with 72 J.cm^{-2} of visible light. The images on the sides show typical images used for quantifying the PI incorporation. (D) Caspase 3 activation in B16-F10 CT and M++++ cells in the dark and after exposure to 72 J.cm^{-2} of visible light. The images on the side are typical for quantifying caspase 3 activation. (*) $p < 0.001$. doi:10.1371/journal.pone.0113266.g003

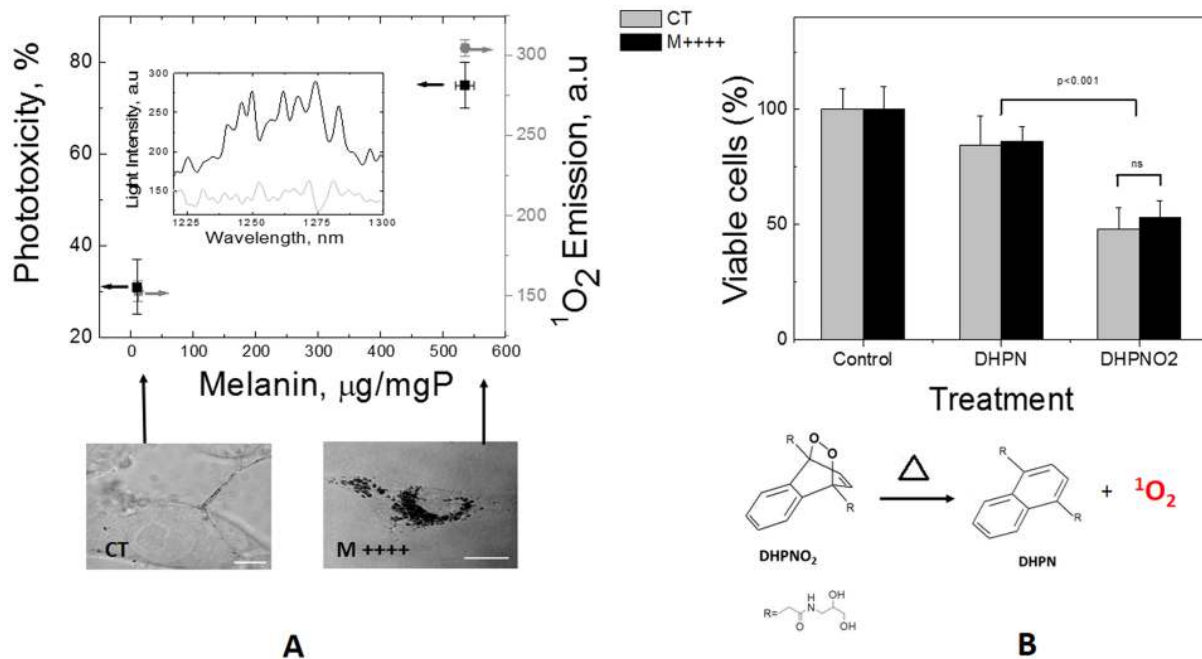


Figure 4. The effects of singlet oxygen on cells. (A) Phototoxicity and integrated emission spectra of the $^1\text{O}_2$ that was generated upon excitation at 532 nm as a function of intracellular melanin production (in μg melanin/mg of total protein, squares) in B16-F10 CT or M++++ cells. Insert: $^1\text{O}_2$ emission spectra from B16-F10 (CT and M++++) cells. The excitation wavelength was 532 nm 5-ns laser pulses at 10 pulses/s and 1 mJ/pulse. (B) Chemical generation of $^1\text{O}_2$ by thermal degradation of DHPNO₂ in CT and M++++ cells. The data are represented as the means \pm SD of three independent experiments, and the results are expressed as the percentage of viable cells compared with that in the control group. (* p <0.05 and *** p <0.001). doi:10.1371/journal.pone.0113266.g004

presence and absence of visible light did not show direct strand breaks, as indicated by the comet assay (data not shown, except for the M++++ cells with visible light irradiation, Fig. 5D). The first three controls (i.e., CT in the presence and absence of light and M++++ in the absence of light) also showed no DNA fragmentation after treatment with Fpg and Endo III (Fig. 5A–C). As mentioned above, the M++++ cells treated with light in the absence of these enzymes did not show DNA fragmentation (Fig. 5D). However, when Fpg or Endo III were applied to M++++ cells, which were irradiated with $6\text{ J}\cdot\text{cm}^{-2}$ of visible light, the comet assay showed a considerable increase in the number of strand breaks (Fig. 5 graph and images E–F), which were absent in the controls (Fig. 5A–D). The presence of strand breaks demonstrated that melanin photosensitization by visible irradiation induces direct oxidative damage to nuclear DNA. The ratio of Fpg- to Endo III-sensitive modifications indicate that oxidative damage in DNA is most likely due to both type II and type I mechanisms [32,33] (Fig. 5, scheme).

Discussion

Although melanin is biosynthesized to protect against UVB, it will damage skin cells in the presence of UVA and visible light. At sufficiently high light doses, melanin causes extensive necroapoptotic cell death. The lower light doses were able to mimic the potential chronic consequences of visible light exposure. Melanin induces the formation of pre-mutagenic DNA lesions (Fpg- and Endo III-sensitive modifications) (Fig. 4, scheme). We also showed that pheomelanin is a more efficient photosensitizer than eumelanin because it generates more $^1\text{O}_2$ and better withstands photobleaching (i.e., it continuously generates $^1\text{O}_2$ for longer periods than does eumelanin).

Therefore, in the presence of melanin, the effects of visible light irradiation do not differ from those of UVA irradiation; consequently, it should be considered with care, and further investigations must be performed to evaluate whether it may be a class I carcinogen [41]. These data indicate a causal relationship between visible light irradiation and the development of genome instability in melanocompetent cells and, consequently, the development of melanoma [17,18,36] and the higher skin cancer prevalence in individuals with red hair [15,37]. Other authors have also concluded that visible light causes effects similar to those of UVA, such as inflammation and ROS production [6,10,17].

The current beliefs regarding the protection of skin against photoinduced damage are similar to those from 30 years ago but with a shift in the problematic wavelength region. In the early 1980 s, photobiologists knew that UVA induced cellular responses [2,3]. However, people were convinced sunbathing with UVB-only protection was safe. The consequences of this strategy are felt today: the resulting deeper skin tumors have a higher prevalence of DNA mutations than those induced by UVA exposure [5,42]. Clearly, visible light affects skin health, but people are encouraged to stay under the sun if they use sufficient amounts of “good sunscreen” (i.e., sunscreens that provide effective protection against UVA and UVB). This recommendation is clearly a mistake because it ignores the effects of visible light, which penetrates more deeply into skin than does UVB and UVA [43], and because it disregards the effects of other wavelength regions, such as the infrared region [44].

The toxicity of visible light raises concerns about other situations in addition to the direct sun phototoxicity to the skin, e.g., clinical protocols that use visible light, such as blue light therapy, in jaundiced babies [45]; indoor tanning [46]; the

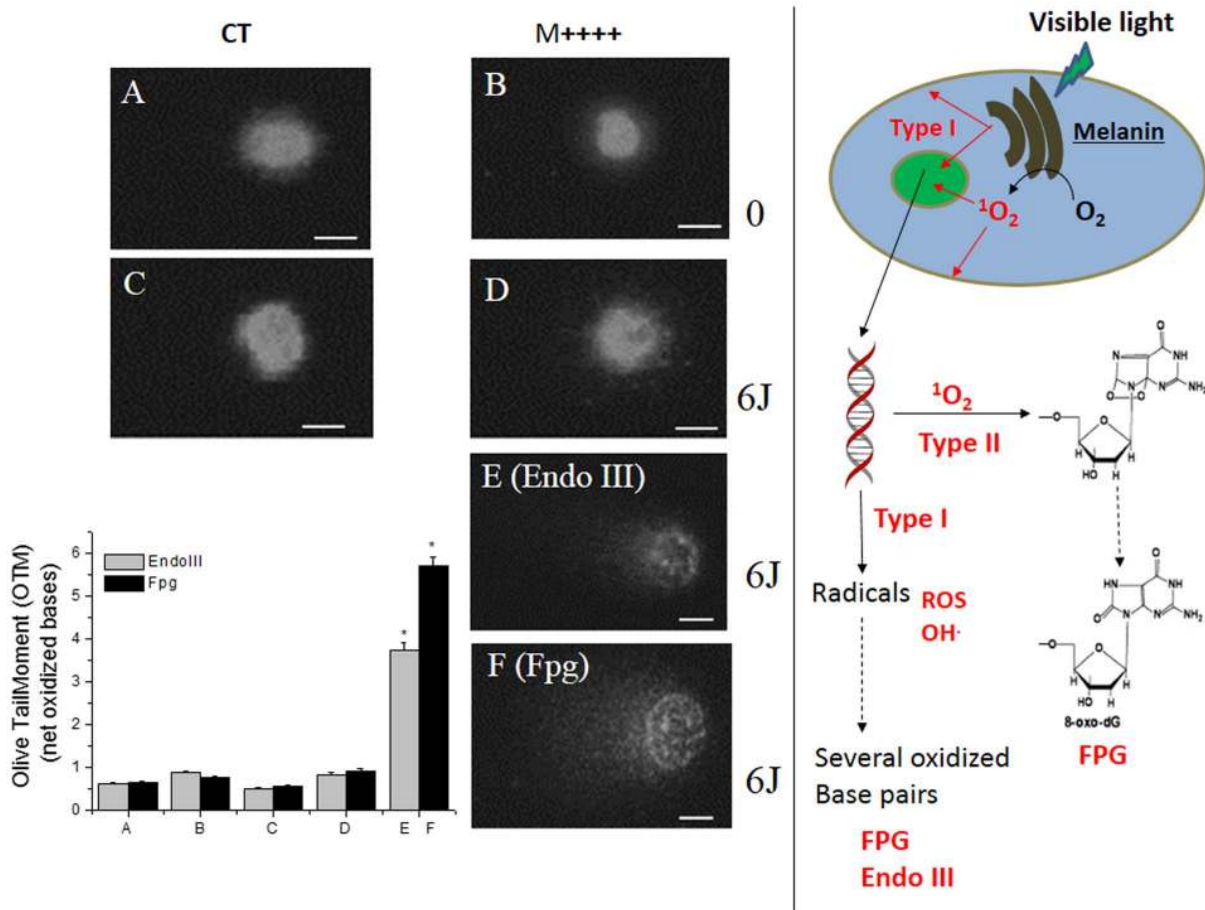


Figure 5. Melanin photosensitization with visible light damages nuclear DNA. (Left) Comet assay. (A) B16-F10 CT cells in the dark after treatment with 0.2 U of Fpg (treatment with Endo III yielded the same results); (B) B16-F10 M++++ cells in the dark after treatment with 0.2 U of Fpg (treatment with Endo III yielded the same results); (C) B16-F10 CT cells exposed to $6 \text{ J} \cdot \text{cm}^{-2}$ of visible irradiation after treatment with 0.2 U of Fpg (treatment with Endo III yielded the same results); (D) B16-F10 M++++ cells exposed to $6 \text{ J} \cdot \text{cm}^{-2}$ of visible irradiation without enzymes; (E, F) the same as D treated with 0.2 U of Endo III (E) and Fpg (F). The graphic on the left shows the quantification of the DNA fragmentation in B16-F10 cells under conditions A through E. (Right) A schematic of melanin photosensitization via type I and type II mechanisms, leading to damage in several biological targets, including the cell membrane and nuclear DNA. The DNA changes are indicated by using the main Fpg and Endo III recognition sites. doi:10.1371/journal.pone.0113266.g005

exposure of eyes to the toxicity of high levels of visible environmental light [47].

In the presence of melanin, visible light generates singlet oxygen and causes direct DNA damage. We hope this information will guide health professionals and the general population to safer interactions with the sun and, specifically, with visible light. We also hope that this information encourages companies to develop new sunscreen products that also provide protection against visible radiation.

Supporting Information

Figure S1 Scheme of the melanin photosensitization mechanisms that generate $^1\text{O}_2$. This $^1\text{O}_2$ can react with the following to form several products: lipids mainly through an ene reaction that forms hydroperoxide, nucleic acids via a guanine residue to form 8-oxo-guanine, and amino acids (the scheme shows the amino acids that are most reactive with $^1\text{O}_2$). The right

side of the scheme shows the thermal decomposition of DHPNO₂, which is also used to generate $^1\text{O}_2$ in the intracellular environment.

(TIF)

Acknowledgments

Prof. S. Ito from Fujita Health University, Japan is acknowledged for providing samples of eumelanin and pheomelanin.

Author Contributions

Conceived and designed the experiments: MSB. Performed the experiments: OCN EA FFF. Analyzed the data: PDM MGM GRM SSME. Contributed reagents/materials/analysis tools: MHM GRM MSB. Contributed to the writing of the manuscript: MSB OCN. Read and provided suggestions and corrections: ASF WKM CP DS FFF SSME GRM EA PDM MHM.

References

1. Yamaguchi Y, Takahashi K, Zmudzka BZ, Kornhauser A, Miller SA, et al. (2006) Human skin responses to UV radiation: pigment in the upper epidermis protects against DNA damage in the lower epidermis and facilitates apoptosis. *FASEB journal: official publication of the Federation of American Societies for Experimental Biology* 20: 1486–1488. Available: <http://www.ncbi.nlm.nih.gov/pubmed/16793869>. Accessed 2014 June 19.
2. Vile GF, Tyrrell RM (1995) UVA radiation-induced oxidative damage to lipids and proteins in vitro and in human skin fibroblasts is dependent on iron and singlet oxygen. *Free radical biology & medicine* 18: 721–730. Available: <http://www.ncbi.nlm.nih.gov/pubmed/7750796>. Accessed 2014 March 15.
3. Berneburg M, Grether-Beck S, Kürten V, Ruzicka T, Briviba K, et al. (1999) Singlet oxygen mediates the UVA-induced generation of the photoaging-associated mitochondrial common deletion. *The Journal of biological chemistry* 274: 15345–15349. Available: <http://www.ncbi.nlm.nih.gov/pubmed/10336420>. Accessed 2013 Dec 23.
4. Cadet J, Douki T (2011) Oxidatively generated damage to DNA by UVA radiation in cells and human skin. *The Journal of investigative dermatology* 131: 1005–1007. Available: <http://www.ncbi.nlm.nih.gov/pubmed/21494240>. Accessed 2014 June 19.
5. Agar NS, Halliday GM, Barnetson RS, Ananthaswamy HN, Wheeler M, et al. (2004) The basal layer in human squamous tumors harbors more UVA than UVB fingerprint mutations: a role for UVA in human skin carcinogenesis. *Proceedings of the National Academy of Sciences of the United States of America* 101: 4954–4959. Available: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=387355&tool=pmcentrez&rendertype=abstract>. Accessed 2014 March 15.
6. Grether-Beck S, Marini A, Jaenicke T, Krutmann J (2014) Photoprotection of human skin beyond ultraviolet radiation. *Photodermatology, photoimmunology & photomedicine* 30: 167–174. doi:10.1111/phpp.12111.
7. Mahmoud BH, Hessel CL, Hamzavi IH, Lim HW (n.d.) Effects of visible light on the skin. *Photochemistry and photobiology* 84: 450–462. Available: <http://www.ncbi.nlm.nih.gov/pubmed/18248499>. Accessed 2014 Jan 19.
8. Kielbassa C, Roza L, Epe B (1997) Wavelength dependence of oxidative DNA damage induced by UV and visible light. *Carcinogenesis* 18: 811–816. Available: <http://www.ncbi.nlm.nih.gov/pubmed/9111219>. Accessed 2014 Jan 19.
9. Kvam E, Tyrrell RM (1997) Induction of oxidative DNA base damage in human skin cells by UV and near visible radiation. *Carcinogenesis* 18: 2379–2384. Available: <http://www.ncbi.nlm.nih.gov/pubmed/9450485>. Accessed 2014 Jan 19.
10. Liebel F, Kaur S, Ruvolo E, Kollias N, Southall MD (2012) Irradiation of skin with visible light induces reactive oxygen species and matrix-degrading enzymes. *The Journal of investigative dermatology* 132: 1901–1907. Available: <http://www.ncbi.nlm.nih.gov/pubmed/22318388>. Accessed 2013 Nov 10.
11. Mahmoud BH, Ruvolo E, Hessel CL, Liu Y, Owen MR, et al. (2010) Impact of long-wavelength UVA and visible light on melanocompetent skin. *The Journal of investigative dermatology* 130: 2092–2097. Available: <http://www.ncbi.nlm.nih.gov/pubmed/20410914>. Accessed 2013 Nov 19.
12. Kolbe L (2012) How much sun protection is needed?: Are we on the way to full-spectrum protection? *The Journal of investigative dermatology* 132: 1756–1757. Available: <http://www.ncbi.nlm.nih.gov/pubmed/22695285>. Accessed 2014 Jan 19.
13. Tadokoro T, Yamaguchi Y, Batzer J, Coelho SG, Zmudzka BZ, et al. (2005) Mechanisms of skin tanning in different racial/ethnic groups in response to ultraviolet radiation. *The Journal of investigative dermatology* 124: 1326–1332. Available: <http://www.ncbi.nlm.nih.gov/pubmed/15955111>. Accessed 2014 Jan 19.
14. Suzukawa AA, Vieira A, Winnischofer SMB, Scalfó AC, Di Mascio P, et al. (2012) Novel properties of melanins include promotion of DNA strand breaks, impairment of repair, and reduced ability to damage DNA after quenching of singlet oxygen. *Free radical biology & medicine* 52: 1945–1953. Available: <http://www.ncbi.nlm.nih.gov/pubmed/22401857>. Accessed 2014 Jan 19.
15. Tadokoro T, Kobayashi N, Zmudzka BZ, Ito S, Wakamatsu K, et al. (2003) UV-induced DNA damage and melanin content in human skin differing in racial/ethnic origin. *FASEB J* 17: 1177–1179. Available: <http://www.ncbi.nlm.nih.gov/pubmed/12692083>.
16. Setlow RB, Grist E, Thompson K, Woodhead AD (1993) Wavelengths Effective in Induction of Malignant-Melanoma. *Proceedings of the National Academy of Sciences of the United States of America* 90: 6666–6670. Available: <http://www.ncbi.nlm.nih.gov/pubmed/12692083>.
17. Wood SR, Berwick M, Ley RD, Walter RB, Setlow RB, et al. (2006) UV causation of melanoma in *Xiphophorus* is dominated by melanin photosensitized oxidant production. *Proceedings of the National Academy of Sciences of the United States of America* 103: 4111–4115. Available: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1449655&tool=pmcentrez&rendertype=abstract>. Accessed 2014 March 15.
18. Chiarelli-Neto O, Pavani C, Ferreira AS, Uchoa AF, Severino D, et al. (2011) Generation and suppression of singlet oxygen in hair by photosensitization of melanin. *Free radical biology & medicine* 51: 1195–1202. Available: <http://www.ncbi.nlm.nih.gov/pubmed/21723388>. Accessed 2013 Nov 18.
19. Ogilby PR (2010) Singlet oxygen: there is indeed something new under the sun. *Chemical Society reviews* 39: 3181–3209. Available: <http://www.ncbi.nlm.nih.gov/pubmed/20571680>. Accessed 2014 April 4.
20. Pierlot C, Aubry JM, Briviba K, Sies H, Di Mascio P (2000) Naphthalene endoperoxides as generators of singlet oxygen in biological media. *Methods in enzymology* 319: 3–20. Available: <http://www.ncbi.nlm.nih.gov/pubmed/10907494>. Accessed 2014 June 19.
21. Haywood RM, Lee M, Andradóttir C (2008) Comparable photoreactivity of hair melanosomes, eumelanin and pheomelanins at low concentrations: low melanin a risk factor for UVA damage and melanoma? *Photochemistry and photobiology* 84: 572–581. Available: <http://www.ncbi.nlm.nih.gov/pubmed/18399925>. Accessed 2014 Jan 19.
22. Ito S, Wakamatsu K (2003) Quantitative analysis of eumelanin and pheomelanin in humans, mice, and other animals: a comparative review. *Pigment Cell Res* 16: 523–531. Available: <http://www.ncbi.nlm.nih.gov/pubmed/12950732>.
23. Uchoa AF, Knox PP, Turchiella R, Seifullina Nk, Baptista MS (2008) Singlet oxygen generation in the reaction centers of *Rhodospirillum rubrum*. *Eur Biophys J* 37: 843–850. Available: <http://www.ncbi.nlm.nih.gov/pubmed/18286272>.
24. Fidler IJ (1975) Biological behavior of malignant melanoma cells correlated to their survival in vivo. *Cancer research* 35: 218–224. Available: <http://www.ncbi.nlm.nih.gov/pubmed/1109790>. Accessed 2014 Oct 9.
25. Boukamp P, Petrussevska RT, Breitkreutz D, Hornung J, Markham A, et al. (1988) Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. *The Journal of cell biology* 106: 761–771. Available: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2115116&tool=pmcentrez&rendertype=abstract>. Accessed 2014 Sept 20.
26. Ralph P, Nakoinz I (1975) Phagocytosis and cytolysis by a macrophage tumour and its cloned cell line. *Nature* 257: 393–394. Available: <http://www.ncbi.nlm.nih.gov/pubmed/1101071>. Accessed 2014 Oct 9.
27. Carey TE, Takahashi T, Resnick LA, Oettgen HF, Old LJ (1976) Cell surface antigens of human malignant melanoma: mixed hemadsorption assays for humoral immunity to cultured autologous melanoma cells. *Proceedings of the National Academy of Sciences of the United States of America* 73: 3278–3282. Available: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=431008&tool=pmcentrez&rendertype=abstract>. Accessed 2014 Oct 9.
28. Brohem CA, Massaro RR, Tiago M, Marinho CE, Jasiulionis MG, et al. (2012) Proteasome inhibition and ROS generation by 4-nerolidylcatechol induces melanoma cell death. *Pigment cell & melanoma research* 25: 354–369. Available: <http://www.ncbi.nlm.nih.gov/pubmed/22372875>. Accessed 2014 Oct 9.
29. Collier AC, Pritsos CA (2003) The mitochondrial uncoupler dicumarol disrupts the MTT assay. *Biochemical pharmacology* 66: 281–287. Available: <http://www.ncbi.nlm.nih.gov/pubmed/12826270>. Accessed 2014 Dec 23.
30. Heo S-J, Ko S-C, Cha S-H, Kang D-H, Park H-S, et al. (2009) Effect of phlorotannins isolated from *Ecklonia cava* on melanogenesis and their protective effect against photo-oxidative stress induced by UV-B radiation. *Toxicology in vitro: an international journal published in association with BIBRA* 23: 1123–1130. Available: <http://www.ncbi.nlm.nih.gov/pubmed/19490939>. Accessed 2013 Nov 30.
31. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry* 72: 248–254. Available: <http://www.ncbi.nlm.nih.gov/pubmed/942051>.
32. Pouget JP, Douki T, Richard MJ, Cadet J (2000) DNA damage induced in cells by gamma and UVA radiation as measured by HPLC/GC-MS and HPLC-EC and Comet assay. *Chemical research in toxicology* 13: 541–549. Available: <http://www.ncbi.nlm.nih.gov/pubmed/10898585>. Accessed 2014 June 19.
33. Sauvaigo S, Petec-Calín C, Caillaud S, Odín F, Cadet J (2002) Comet assay coupled to repair enzymes for the detection of oxidative damage to DNA induced by low doses of gamma-radiation: use of YOYO-1, low-background slides, and optimized electrophoresis conditions. *Analytical biochemistry* 303: 107–109. Available: <http://www.ncbi.nlm.nih.gov/pubmed/11906159>. Accessed 2014 June 19.
34. Olive PL, Banáth JP (2006) The comet assay: a method to measure DNA damage in individual cells. *Nature protocols* 1: 23–29. Available: <http://www.ncbi.nlm.nih.gov/pubmed/17406208>. Accessed 2014 Jan 10.
35. Hearing VJ (2000) The melanosome: the perfect model for cellular responses to the environment. *Pigment cell research*/sponsored by the European Society for Pigment Cell Research and the International Pigment Cell Society 13 Suppl 8: 23–34. Available: <http://www.ncbi.nlm.nih.gov/pubmed/11041354>. Accessed 2014 June 19.
36. Lin JY, Fisher DE (2007) Melanocyte biology and skin pigmentation. *Nature* 445: 843–850. Available: <http://www.ncbi.nlm.nih.gov/pubmed/17314970>. Accessed 2014 Jan 18.
37. Rees JL (2003) Genetics of hair and skin color. *Annual review of genetics* 37: 67–90. Available: <http://www.ncbi.nlm.nih.gov/pubmed/14616056>. Accessed 2014 Jan 19.
38. Beltrán-García MJ, Prado FM, Oliveira MS, Ortiz-Mendoza D, Scalfó AC, et al. (2014) Singlet molecular oxygen generation by light-activated DHN-melanin of the fungal pathogen *Mycosphaerella fijiensis* in black Sigatoka disease

- of bananas. *PLoS one* 9: e91616. Available: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3960117&tool=pmcentrez&rendertype=abstract>. Accessed 2014 June 19.
39. Russo MT, Blasi MF, Chiera F, Fortini P, Degan P, et al. (2004) The oxidized deoxynucleoside triphosphate pool is a significant contributor to genetic instability in mismatch repair-deficient cells. *Molecular and cellular biology* 24: 465–474. Available: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=303369&tool=pmcentrez&rendertype=abstract>. Accessed 2014 Jan 19.
 40. Hatahet Z, Kow YW, Purmal AA, Cunningham RP, Wallace SS (1994) New substrates for old enzymes. 5-Hydroxy-2'-deoxycytidine and 5-hydroxy-2'-deoxyuridine are substrates for *Escherichia coli* endonuclease III and formamidopyrimidine DNA N-glycosylase, while 5-hydroxy-2'-deoxyuridine is a substrate for uracil DNA N-glycos. *The Journal of biological chemistry* 269: 18814–18820. Available: <http://www.ncbi.nlm.nih.gov/pubmed/8034633>. Accessed 2014 Jan 19.
 41. Dahle J, Kvam E (2003) Induction of delayed mutations and chromosomal instability in fibroblasts after UVA-, UVB-, and X-radiation. *Cancer research* 63: 1464–1469. Available: <http://www.ncbi.nlm.nih.gov/pubmed/12670891>. Accessed 2014 Jan 19.
 42. Halliday GM, Agar NS, Barnetson RSC, Ananthaswamy HN, Jones AM (2005) UV-A fingerprint mutations in human skin cancer. *Photochemistry and photobiology* 81: 3–8. Available: <http://www.ncbi.nlm.nih.gov/pubmed/15335275>. Accessed 2014 March 15.
 43. Mahmoud BH, Hexsel CL, Hamzavi IH, Lim HW (2007) Effects of visible light on the skin. *Photochemistry and photobiology* 84: 450–462. Available: <http://www.ncbi.nlm.nih.gov/pubmed/18248499>. Accessed 2014 March 6.
 44. Schieke SM, Schroeder P, Krutmann J (2003) Cutaneous effects of infrared radiation: from clinical observations to molecular response mechanisms. *Photodermatology, photoimmunology & photomedicine* 19: 228–234. Available: <http://www.ncbi.nlm.nih.gov/pubmed/14535893>. Accessed 2014 March 16.
 45. Opländer C, Hidding S, Werners FB, Born M, Pallua N, et al. (2011) Effects of blue light irradiation on human dermal fibroblasts. *Journal of photochemistry and photobiology B, Biology* 103: 118–125. Available: <http://www.ncbi.nlm.nih.gov/pubmed/21421326>. Accessed 2014 Jan 19.
 46. Youssef PN, Sheibani N, Albert DM (2011) Retinal light toxicity. *Eye (London, England)* 25: 1–14. Available: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3144654&tool=pmcentrez&rendertype=abstract>. Accessed 2014 Jan 19.
 47. Fisher DE, James WD (2010) Indoor tanning—science, behavior, and policy. *The New England journal of medicine* 363: 901–903. Available: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3951814&tool=pmcentrez&rendertype=abstract>. Accessed 2014 March 16.