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UV Irradiation Affects Melanocyte Stimulatory Activity and Protein Binding of Piperine

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ABSTRACT

Piperine, the major alkaloid of black pepper (*Piper nigrum* L.; Piperaceae), stimulates melanocyte proliferation and dendrite formation *in vitro*. This property renders it a potential treatment for the skin depigmentation disorder vitiligo. However, piperine does not stimulate melanin synthesis *in vitro*, and treatments based on this compound may therefore be more effective with concomitant exposure of the skin to ultraviolet (UV) radiation or sunlight. The present study investigated the effect of UVA and simulated solar radiation (SSR) on the chemical stability of piperine, its melanocyte stimulatory effects and its ability to bind protein and DNA. Chromatographic and spectroscopic analysis confirmed the anticipated photoisomerization of irradiated piperine and showed the absence of any hydrolysis to piperinic acid. Isomerization resulted in the loss of ability to stimulate proliferation of a mouse melanocyte cell line, and to bind to human serum albumin. There was no evidence of DNA binding by piperine either before or after irradiation, showing the absence of photoadduct formation by either piperine or its geometric isomers. This is unlike the situation with psoralens, which form DNA adducts when administered with UVA in treating skin diseases. The present study suggests that exposure to bright sunlight should be avoided both during active application of piperine to the skin and in the storage of piperine products. If UVA radiation is used with piperine in the treatment of vitiligo, application of the compound and irradiation should be staggered to minimize photoisomerization. This approach is shown to effectively induce pigmentation in a sparsely pigmented mouse strain.

INTRODUCTION

We have previously reported that piperine (Fig. 1), the major alkaloid found in the fruit of black pepper (*Piper nigrum* L.; Piperaceae) (1), stimulates the replication of melanocytes and induces the formation of melanocytic dendrites *in vitro* (2,3). Melanocytes are pigment-producing dendritic cells located within the basal layer of the epidermis and in the matrix of hair follicles. Melanin is synthesized within organelles known as melanosomes, which are transferred through melanocytic dendrites to epidermal keratinocytes (4), resulting in the observed pigmentation of mammalian skin. The stimulatory effects of piperine on melanocyte proliferation and dendricity render it a potential treatment for vitiligo, a skin disorder characterized by depigmented lesions (4). Melanocytes have been shown to be absent, or present in very small numbers, in vitiligo lesions apart from reservoirs found in the hair follicles (5–7). Piperine is expected to cause the repopulation of vitiligo patches through a stimulatory effect on perilesional and follicular melanocytes. However, piperine does not stimulate melanin synthesis in melanocytes *in vitro* (2,3). A potential treatment option is to expose the skin to ultraviolet radiation (UVR) along with piperine in order to induce pigmentation in the new melanocytes. UVR can act directly on melanocytes to increase skin pigmentation or indirectly through the release of keratinocyte-derived factors such as cytokines, eicosanoids, growth factors, nitric oxide or melanotropic hormones (8–10).

UVA irradiation (320–400 nm) is already used in the treatment of vitiligo, in conjunction with orally or topically administered psoralens (PUVA therapy—psoralens plus UVA) (11). Psoralens form monofunctional and bifunctional photoadducts with cellular DNA upon exposure to UVA; this process stimulates melanocyte replication and melanogenesis (12,13). Solar simulated radiation (SSR) plus 5-methoxypsoralen (5-MOP) has been used to induce human pigmentation (12). 8-MOP, which is more commonly used in clinical practice, has been shown to induce epidermal melanogenesis in human (14) and murine (15) skin when administered with UVA.

Unlike psoralens (12–15), piperine is able to induce melanocyte proliferation *in vitro* even in the absence of UVR, possibly by a mechanism involving stimulation of protein kinase C (2). A potential complicating factor in the concomitant use of UVR to induce melanogenesis in these cells is that piperine is known to undergo photoisomerization at the double bonds on exposure to UVR (350 nm) (16) and sunlight (17). Although a number of structural analogs of piperine have been found to share its melanocyte stimulatory effects (3), the activity of its geometric isomers has not previously been examined.

The present study investigates the effect of UVR on the *in vitro* and *in vivo* stimulatory effects of piperine on melanocytes and on

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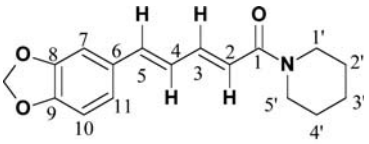
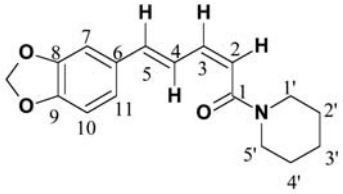
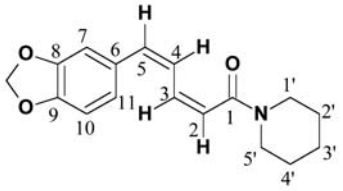


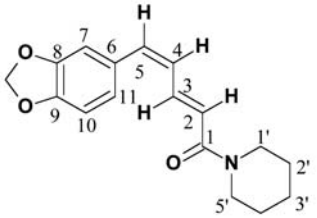
Piperine [*E,E*-(trans-trans)-piperine]



Isopiperine [*Z,E*-(cis-trans)-piperine]



Chavicine [*Z,Z*-(cis-cis)-piperine]



Isochavicine [*E,Z*-(trans-cis)-piperine]

Figure 1. Molecular structure of piperine (1-2*E,4E*-piperinoyl-piperidine) and its three geometric isomers.

its ability to interact with proteins and DNA. The involvement of DNA binding either before or after irradiation in the action of piperine has not previously been investigated. These studies are relevant to the design of preclinical and clinical studies on the efficacy of piperine with UVR as a stimulant for skin repigmentation in vitiligo.

MATERIALS AND METHODS

Chemicals. All chemicals and reagents were purchased from Sigma-Aldrich Co. Ltd. (Irvine, UK) unless stated otherwise. Piperinic acid was prepared in our laboratory by hydrolysis of piperine (24).

Radiation sources and dosimetry for in vitro *experiment simulated solar radiation (SSR)*. SSR was obtained from a Solar Simulator (Oriel, Stratford, CT) with the use of a 1-kW xenon arc lamp (ORC Lighting Products, Azusa, CA) in conjunction with a quartz collimator, quartz lens and a Schott WG320 filter (1 mm thick). Irradiance was routinely measured with a wideband thermopile radiometer (Medical Physics, Dryburn Hospital, Durham, UK) calibrated with a double-monochromator spectroradiometer (DM150, Bentham, Reading, UK) which had been calibrated against a UK National Physics Laboratory (NPL) standard lamp. Irradiance was 12 mW cm^{-2} scanned between 280 and 400 nm (UVB 280–320 nm accounting for 8.5% of total output) at 11 cm, which was the distance to the test sample. The emission spectrum of this source has been previously reported (25).

Broad-spectrum UVA. Broad spectrum UVA-I (340–400 nm) was obtained from a UVASUN2000 (Mutzhas, Munich, Germany), which was housed on a custom-built trolley allowing irradiation from above. Two fans blew across the irradiation area to provide cooling. Routine irradiance was measured with an IL 442 radiometer (International Light, Newburyport, MA) and calibrated in the same way as the thermopile used for SSR. Irradiance was 74 mW cm^{-2} (over the spectral range 340–400 nm) at the test surface (a distance of 10 cm); of the total output, 98.88% was UVA-I (340–400 nm), 1.11% was UVA-II (320–340 nm) and 0.01% was UVB (280–320 nm). The emission spectrum of this source has been previously reported (25).

Irradiation of piperine solutions. Piperine was dissolved in CD_3OD (40 mg/mL). This solution (2 mL) was carefully pipetted into petri dishes placed on ice to minimize evaporation. Petri-dish lids were removed prior to irradiation with either UVA or SSR. After irradiation, solutions were collected and any volume lost by evaporation made up to 2 mL with CD_3OD. The control solutions (nonirradiated) were covered with aluminum foil and kept at 4°C.

Analysis of irradiated solutions. ^1H NMR was recorded using a Bruker (360 MHz) NMR spectrometer. Reversed-phase HPLC analysis of irradiated and unexposed piperine was performed with the use of an Alltech Adsorbosphere 4.6 mm × 25 cm RP-C18 (10 μm) column with a 10 mm C18 guard column with a LDC Analytical 3100 pump and a HP 3390A Integrator connected to a spectromonitor 3100 UV detector (LDC Analytical, Riviera Beach, FL). Analyses were performed isocratically with a mobile phase containing 60% water, 40% acetonitrile; 1 mL/min. An injection volume of 10 μL was used; the eluent was monitored at 348 nm. The retention time of piperine in this system was 12.7 min. LC-MS analysis was undertaken with the use of an Alltech Adsorbosphere 4.6 mm × 25 cm RP-C18 (10 μm) column in a ThermoFinnigan Surveyor LC system directly coupled to ThermoFinnigan LCQ DECA XP ion-trap mass spectrometer operating in the electrospray positive mode. UV spectra were obtained from the ThermoFinnigan Surveyor Photo Diode Array (PDA) detector associated with the LC-MS system. Analyses were performed isocratically with a mobile phase comprising 85% acetonitrile, 15% water. An injection volume of 10 μL was used; the samples were eluted at a flow rate of 0.2 mL/ min and monitored at 348 nm on the PDA for piperine. The retention time of piperine in this system was 11.7 min.

Cell culture experiments

Stock cultures. Melan-a cells are an immortal pigmented mouse cell line, cultured from epidermal melanoblasts from embryos of inbred C57BL mice (26). Subconfluent to nearly confluent melan-a cultures (passage number 26–31) were used in this study. Cell cultures were maintained in culture flasks in supplemented RPMI 1640 growth medium with 200 nM TPA, trypsinized, harvested and resuspended for experiments in the same medium without TPA, as described earlier (2,3).

Preparation of microplates with melan-a cell suspension. Melan-a cells were inoculated (100 μL; 6×10^3 cells per well) with a repeater pipetter into 96-well microtiter plates (Nunc, Cambridge) and incubated at 37°C in a 10% CO_2, 90% air humidified atmosphere for 4 h. Piperine was dissolved in methanol and the solutions were sterilized by filtration (pore size 0.2 μm) and then diluted with the cell culture medium to give a final stock solution of 30 μM piperine and a nontoxic concentration of methanol. Each plate was subdivided into sections each consisting of two adjacent columns of 6 wells each for piperine (10 μM; 50 μL of stock solution) or control (50 μL medium only), with a gap of 2 empty columns in between each section.

Irradiation and culture of melan-a cells. The plates, placed on ice, were positioned under the UVA and SSR irradiation sources. In order to maintain sterility of the cell cultures, irradiations needed to be carried out with the microplate lid in place. The lid reduced the UVB and UVA irradiances of the SSR source by about 45 and 30%, respectively, and the UVA irradiance of the UVA source by about 20%. The doses given below and in Figs. 6 and 7 are not corrected for the effects of the lid, so that comparisons can be made with Fig. 3B. Microplates were irradiated with UVA doses ranging from 0 to 124 J cm^{-2} with the use of the Mutzhas UVASUN2000 broadband UVA source. This represents a dose of less than 2 minimal erythema doses (MED) (27) for sun-sensitive skin Type II after correction for absorption by the 96-well-plate lid. Different doses were achieved by exposing particular sections of the plate (each consisting of one row of

piperine exposed cells and one row of control cells) for different time periods (0, 7, 13, 22 or 28 minutes; $n = 6$). Unexposed areas were covered with a piece of cardboard. Another set of microplates was irradiated with SSR doses ranging from 0 to 15 J cm^{-2} with the use of the solar simulator for 0, 5, 7, 15 or 21 min ($n = 6$). This represents doses of about 1.5 MEDs for skin Type II (filter 2 data [27]) after corrections for the effect of the microplate lid. All plates were incubated at 37°C in a 10% CO_2, 90% air humidified atmosphere incubator for 4 days.

Measurement of cell proliferation. The basic protocol was based on the assay developed by Skehan *et al*. (28) with modifications (29). Briefly, after 4 days incubation cells were fixed with the use of cold trichloroacetic acid solution, incubating at 4°C for 1 h. After washing with tap water to remove acid, medium and dead cells, plates were dried in air and SRB dye was added. At the end of the staining period (30 min) unbound SRB was removed by washing with acetic acid and air drying. Cell-bound dye was solubilized in Tris [tris(hydroxymethyl)aminomethane] base and absorbance was read at 550 nm in a microplate spectrophotometer (Spectromax 190 Molecular Devices, Softmax Pro Version 2.2.1, 1998).

Statistical analysis. Statistical significance of differences between the number of melanocytes in control and test incubations was determined with the use of one-way ANOVA followed by a Dunnett's *t* test.

DNA and human serum albumin (HSA) binding assay

DNA and HSA binding of piperine was monitored using a 5 : 1 DNA base pair: drug or protein: drug molar ratio, using piperine (100 μM) and DNA or HSA (500 μM), all made up in 1% methanolic phosphate buffer (pH 7.4). Mixtures were made of piperine with buffer, DNA solution, or HSA solution. Pure solutions of piperine, DNA and HSA and mixtures were divided into three sets with one set left unexposed, the second set exposed to UVA for 5 min (22 J cm^{-2}) and the third set exposed to SSR for 21 min (15 J cm^{-2}). These solutions were then stored in a refrigerator for 3 days prior to circular dichroism, CD (Jasco J-600 spectropolarimeter) and linear dichroism, LD (Jasco J-720 spectropolarimeter) analysis. The irradiated samples were analyzed by UV spectroscopy (Perkin-Elmer Lambda-2 UV/VIS spectrometer) immediately after irradiation and after 3 days' storage in a refrigerator. No changes were found to have occurred during the storage period (results not shown). CD spectroscopy was performed using a CD cell of 1 cm path length. LD analysis was performed to validate CD experiments on piperine binding to DNA.

In vivo evaluation of pigmentation induced by piperine and /or UVR

Animals. Male inbred HRA.HRII-c/+/Skh hairless pigmented mice, age-matched (8–16 weeks old), were used in each study. This line, congenic with albino inbred HRA/Skh mice, segregates into albino and pigmented phenotypes and was developed by Dr. P. Forbes, Temple University Centre for Photobiology (bred by the Biological Services Division, KCL, University of London, and the Rayne Institute, St. Thomas's Hospital, London).

Treatment groups. Mice ($n = 4$ per group) were treated with (A) dimethylsulfoxide (DMSO) for 9 weeks, (B) piperine (175 mM) dissolved in DMSO for 9 weeks, (C) piperine in DMSO for 9 weeks with UVR from Weeks 5–9, or (D) UVR only for 5 weeks. Piperine solution and DMSO were applied with a micropipette (100 μL) on dorsal skin twice a day (weekdays) with an interval of 5–6 h between applications. UVR was administered as described below. For group (C) the irradiations were carried out every Monday, Wednesday and Friday immediately prior to the first daily application of piperine to avoid photodegradation of piperine.

UV irradiation and dosimetry. The UVR source was a bank of eight Bellarium SA-1-12-100 W fluorescent tubes (Wolff, Erlangen, Germany). This UVR source emits 4.1% UVB (280–320 nm) and 95.8% UVA, but the UVB accounts for the 71.5% erythemally effective energy when biologically weighted with the human erythema spectrum (30,31). Irradiations were carried out in a custom-built unit with ventilation, temperature and humidity controls. The irradiance was monitored daily immediately before irradiations with International Light radiometer (IL 422A; Newburyport, MA) equipped with UVR sensors. The radiometer was calibrated for the source, as described before (30). Irradiance at mouse level was typically about 0.16 mW/cm^2. Animals were unrestrained in metal cages and irradiated with a dose of 354 mW cm^{-2} (30) that was further confirmed to be subinflammatory from a single exposure (increase in skin folding thickness <10%; data not shown). Irradiations lasted for a maximum of 1 h. The position of cages was systematically rotated to ensure even UVR exposure.

Assessment of pigmentation. Pigmentation was assessed visually by an investigator blinded to the treatment that the animals had received, with the following scoring system: 0 = no pigmentation; 1 = first signs of pigmentation (spots); 2 = light brown; 3 = medium brown; 4 = dark brown; 5 = black. Scores obtained at the end of each week (Friday) are shown in Fig. 11.

Statistical analysis. Differences between treatment groups across the entire treatment period were compared by the Mann–Whitney *U*-test.

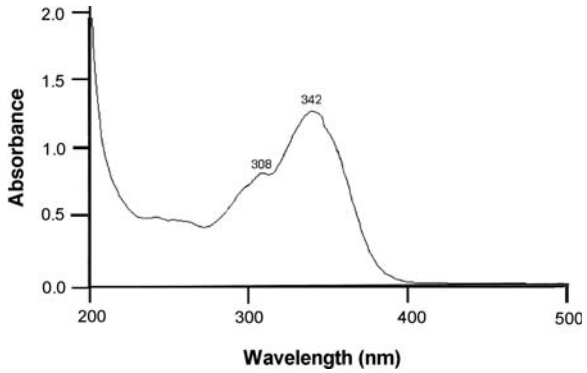


Figure 2. Ultraviolet absorption spectrum of piperine (0.06 mM) in methanol.

RESULTS AND DISCUSSION

Piperine is 1-2*E*,4*E*-piperinoyl-piperidine (Fig. 1) with two trans double bonds in the chain connecting the methylenedioxyphenyl and piperidine groups. Figure 2 shows the UV spectrum of this compound. Possible geometric isomers (Fig. 1) are chavicine (2*Z*, 4*Z*; cis-cis), isopiperine (2*Z*, 4*E*; cis-trans) and isochavicine (2*E*, 4*Z*; trans-cis). Conversion of piperine to these geometric isomers has been reported following exposure to UVR (λ_{max} 350 nm [16,18] or 366 nm [19]) and sunlight (17). In early work (16), chavicine, and later isochavicine, were noted as being the major product of piperine's photoisomerization. However, later studies (17–19) have shown that all three isomers form from piperine, their ratio being dependent on the length of exposure to irradiation. Chavicine appears to be the last isomer to be produced (17,18) and was the dominant isomer after 24 h exposure to sunlight (17).

In the present study, irradiated and unirradiated solutions of piperine in methanol were compared by high-performance liquid chromatography (HPLC), liquid chromatography coupled to mass spectrometry (LC-MS) and UV and ^{1}H NMR (nuclear magnetic resonance) spectroscopy. An HPLC chromatogram of piperine irradiated with UVA (124 J cm^{-2}) is shown in Fig. 3A. The main peak at R_t 12.7 min corresponds to piperine, whereas the peak eluting just before piperine (R_t 12.0 min) represents one or more photoproducts of piperine. Figure 3B shows that the relative area of the photoproduct(s) at 12.0 min increased with increasing UVR dose. No peak for piperinic acid (retention time of standard = 6.3 min) was observed in HPLC analysis, showing that no hydrolysis had taken place at the amide function. Individual isomers were not resolved on this HPLC system, but methods for their baseline separation have been reported elsewhere (17).

Tandem LC-MS (LC-MS^n) analysis of the two HPLC peaks revealed that both were comprised of substances with identical mass spectra. ESI-MS *m/z* in positive-ion mode for the piperine peak gave a quasimolecular ion at *m/z* 286 (100%) $[M + H]^+$. Further fragmentation (MS^2) of this ion gave *m/z* 201, which was then further fragmented (MS^3) to give *m/z* 173, 171, 143 and 115. The photoproduct HPLC peak gave virtually identical ions in terms

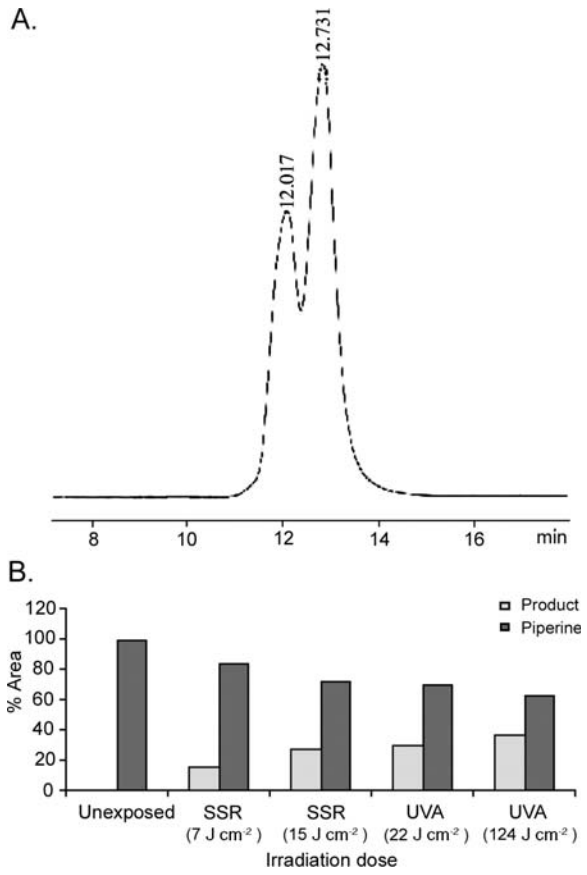


Figure 3. (A) Partial HPLC chromatogram of a methanolic solution of piperine after exposure to UVA (124 J cm^{-2}). The peak at 12.7 min corresponds to that obtained with unexposed piperine, whereas that at 12.0 min is present only in irradiated samples. HPLC conditions as in the text. (B) Relative areas of the HPLC peaks seen at 12.7 min (piperine) and 12.0 min (product) when piperine is exposed to different levels of radiation. The two peaks were not completely resolved, and are not composed purely of piperine or its photoproducts. Areas were obtained by integrating the peak areas on either side of a vertical line dropped from the valley between the two peaks.

of both *m/z* value and relative abundance. Mass spectra are reported to be identical for the four isomeric compounds (19,20). The MS fragments obtained in this study were in excellent agreement with the literature for piperine and its isomers (19).

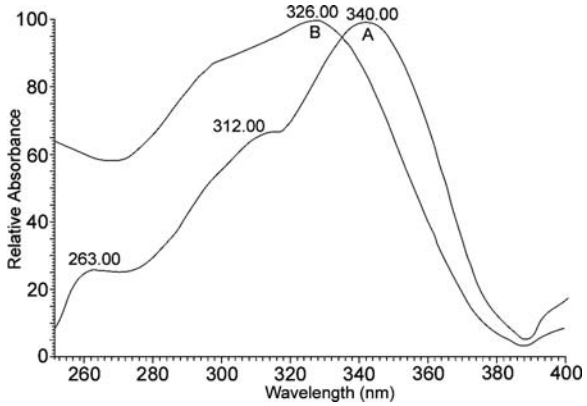


Figure 4. UV spectra of HPLC peaks corresponding to piperine (A) and photoproduct (B) after exposure to UVA (124 J cm^{-2}). Spectra were obtained using a photodiode array detector attached to the HPLC system.

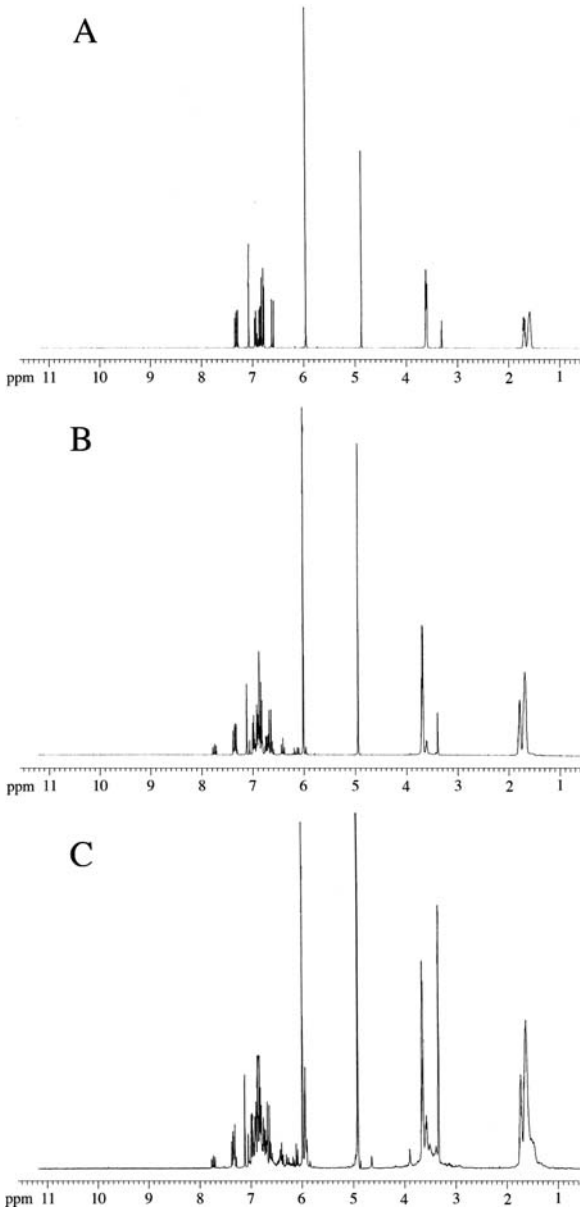


Figure 5. ^{1}H-NMR spectra of piperine (A), piperine after exposure to SSR at 22 J cm^{-2} (B) and piperine after exposure to UVA at 112 J cm^{-2} (C). Spectra were recorded in CD_3OD.

LC-MS data therefore confirm that the photomodified product consists of one or more geometric isomers of piperine.

Piperine isomers vary in their UV absorption λ_{max} values. Reported values (17,19,20) range as follows: piperine 340–343 nm, isochavicine 330–336 nm, isopiperine 332–335 nm and chavicine 317–321 nm. UV analysis of the peaks observed in HPLC, using diode array detection coupled to the LC-MS instrument, showed a λ_{max} = 326 nm for the photomodified product peak as compared to 340 nm for piperine (Fig. 4). This clearly shows the presence of chavicine, which is the only isomer with a λ_{max} value lower than 330 nm. The presence of other isomers is likely to have caused the λ_{max} to shift to a slightly higher wavelength than the range reported for chavicine (317–321 nm).

The ^{1}H-NMR spectra of piperine in CD_3OD (deuterated methanol) before and after irradiation are shown in Fig. 5A–C.

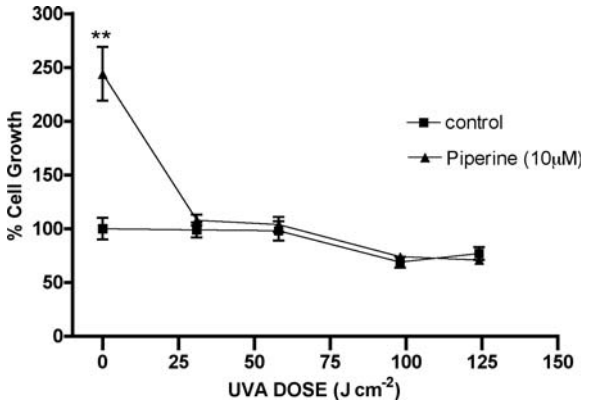


Figure 6. Effect of UVA irradiation on cell growth in the presence or absence (control) of 10 μM piperine. Cell growth is expressed as a percentage of cell growth in cultures unexposed to radiation or piperine. $^{**}P < 0.01$ when compared to control incubation at the same radiation dose (one-way ANOVA followed by Dunnett's *t*-test). Note that doses are not corrected for the absorption of the 96-well-plate lid, which absorbs about 20% of the UVA.

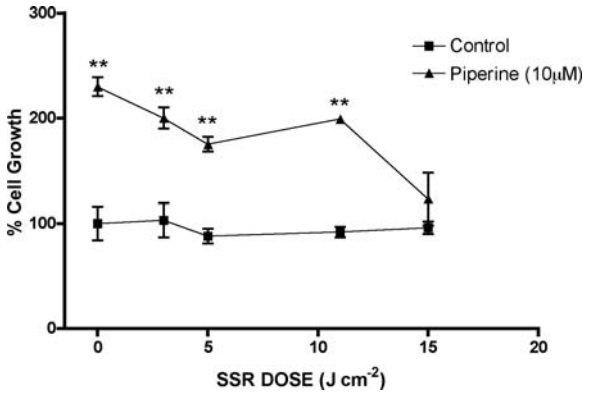


Figure 7. Effect of SSR irradiation on cell growth in the presence or absence (control) of 10 μM piperine. Cell growth is expressed as a percentage of cell growth in cultures unexposed to radiation or piperine. $^{**}P < 0.01$ when compared to control incubation at the same radiation dose (one-way ANOVA followed by Dunnett's *t*-test). Note that doses are not corrected for the absorption of the 96-well-plate lid, which absorbs about 30% of the UVA and 45% of the UVB.

The ^{1}H NMR (CD_3OD, 360MHz) signals for piperine are at δ 7.31(1H, ddd, $J_{3,2} = 14.7$ Hz, $J_{3,4} = 8.2$ Hz, $J_{3,5} = 2.0$ Hz, **H-3**), 7.07 (1H, d, $J_{7,11} = 1.6$ Hz, **H-7**), 6.95 (1H, dd, $J_{11,10} = 8.0$ Hz, $J_{11,7} = 1.6$ Hz, **H-11**), 6.88 (1H, dd, $J_{4,5} = 15.2$Hz, $J_{4,3} = 8.2$ Hz, **H-4**), 6.80 (1H, d, $J_{5,4} = 15.2$ Hz, **H-5**), 6.78 (1H, d, $J_{10,11} = 8.0$ Hz, **H-10**), 6.60 (1H, d, $J_{2,3} = 14.7$ Hz, **H-2**), 5.95 (2H, s, **OCH_2O**), 4.90 (solvent), 3.60 (4H, br, **H-1′**, **H-5′**), 1.70 (2H, m, **H-3′**), 1.60 (4H, m, **H-2′4′**). This spectrum is in agreement with the literature (17,19,20), minor differences in δ values being due to the use of $CDCl_3$ (deuterated chloroform) as solvent in the earlier studies.

The ^{1}H-NMR spectra of irradiated piperine solutions (Fig. 5B,C) showed significant differences from that of piperine, which were more pronounced in the UVA- than in the SSR-irradiated sample. The spectra were very similar to that previously reported for piperine samples exposed to sunlight (17). In both cases (Fig. 5B,C), the most significant changes are observed in the 6–8 ppm region, representing the double-bond and phenyl protons, indicative of changes in the configuration of the double bond. In both irradiated samples, the strong signal at about 5.98 ppm is still apparent, indicating that the methylenedioxyphenyl group is intact, although there is now some overlap with the double-bond signals. Three previous studies (17,19,20) discuss comparative ^{1}H-NMR spectra of the four isomers. A doublet assignable to **H-2** is found at around δ 6.0 in the ^{1}H-NMR spectra of isopiperine and chavicine, both of which have a cis arrangement of the **H-2, H-3** double bond), whereas this proton is seen at around δ 6.5 for piperine or isochavicine. Isochavicine is the only isomer reported to have a signal **(H-3)** downfield of δ 7.5 (15,17,18), and the **H-4** signal appears uniquely in the δ 6.3–6.4 region for this isomer. New signals around δ 6 were seen in the two irradiated samples of piperine (Fig. 5B,C), showing that either chavicine or isopiperine, or both, are present in the mixture. The irradiated solutions also show a new double doublet at δ 6.4 and multiplet at around δ 7.75, confirming the presence of isochavicine. ^{1}H NMR analysis therefore confirms that UVA and, to a lesser extent, SSR cause the photoisomerization of piperine in the present study. The proportion of isochavicine in the mixture can be obtained from a comparison of the integration value for the isochavicine **H-3** signal at δ 7.75 (1.08 SSR; 0.42 UVR) with the value obtained for the six piperidine protons at **H-2′, 3′, 4′** appearing at δ < 2.0 (29.84 SSR; 23.09 UVR). Interestingly, there is less isochavicine in the UVR-treated (11%) than the SSR-treated (22%) sample.

In cell cultures unexposed to irradiation (Figs. 6 and 7), piperine stimulated melanocyte proliferation and dendrite formation (Fig. 8) as expected from earlier studies (2,3). However this effect was lost on exposure to UVA (Fig. 6) or SSR (Fig. 7). For SSR, the effect

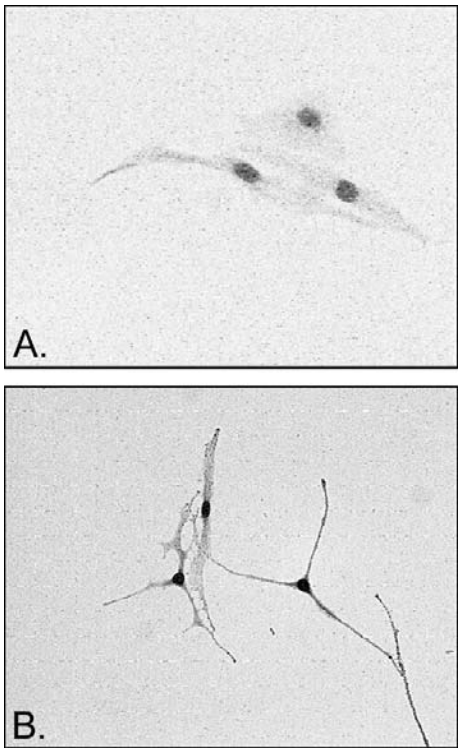


Figure 8. Melan-a cells grown in control culture medium show a bipolar morphology (A). Exposure to piperine (10 μM) induces dendricity in these cells (B).

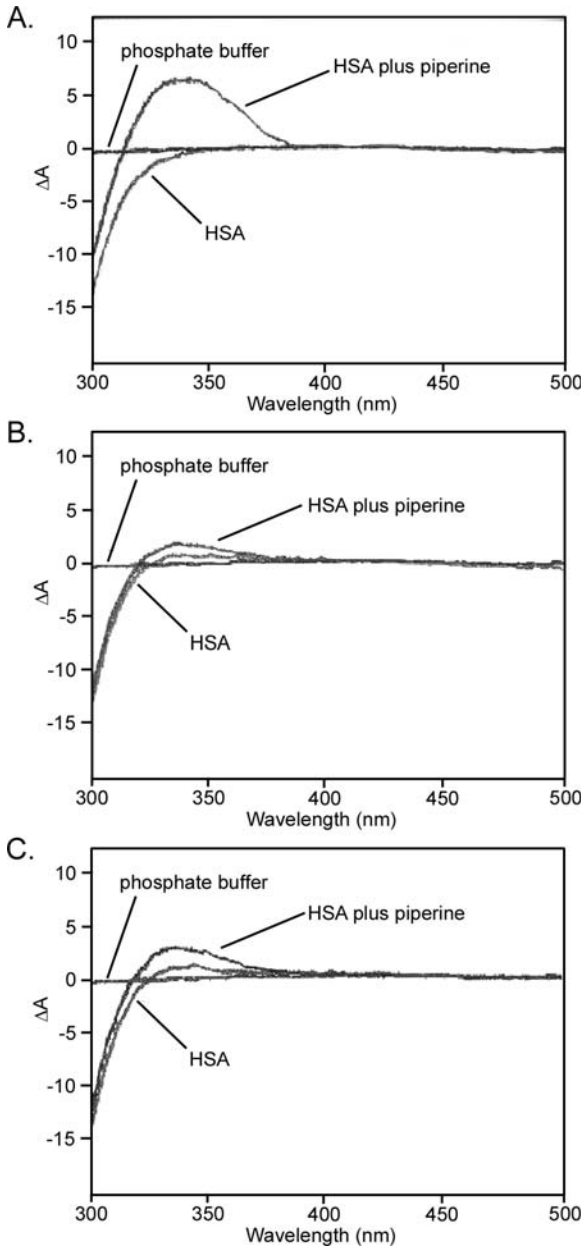


Figure 9. CD study of piperine's interaction with HSA, before exposure to irradiation (A), after exposure to UVA (22 J cm^{-2}) (B), and after exposure to SSR (15 J cm^{-2}) (C).

was radiation dose dependent, probably due to lower levels of isomerization with this form of radiation as compared to the doses used with UVA alone. UVA has previously been shown to cause DNA damage in cultured human melanocytes *in vitro* (21–23). However, in the present study, this does not account for the observed decline in cell growth in irradiated, piperine-treated cultures. Control cells exposed to SSR radiation at all test doses grew as well as unexposed cells (Fig. 7), whereas UVA-exposed cells showed a small decline in growth only at radiation doses of 98 J cm^{-2} and above, whereas the stimulatory effect of piperine was lost even at 31 J cm^{-2} of UVA (Fig. 6).

We have previously reported that a large number of analogues of piperine can stimulate melanocyte proliferation (3), implying that the structural requirements for activity may not be very stringent. However, the present study shows that the configuration around the double bonds in the 1-piperinoyl-piperidine molecule may have a profound effect on activity. The isomerization induced by UVA and SSR radiation results in a loss of activity of the molecule in proportion to the radiation dose received. A change from a trans, trans configuration to one involving cis bonds would lead to a considerable alteration in the overall shape of the molecule. Piperine would be expected to have a relatively linear structure, whereas the other three isomers, each containing at least one cis bond, are more folded (Fig. 1). It is of note that tetrahydropiperine, in which the two double bonds are replaced by a saturated 4-carbon aliphatic chain is also active (3), presumably because it can adopt a linear conformation similar to piperine.

Potential binding of piperine to protein and DNA before and after irradiation was investigated using circular dichroism (CD). Piperine is not optically active, but if bound to a chiral substance such as human serum albumin (HSA) or DNA, optical activity is induced and a signal will be observed in the CD spectrum associated with piperine's UV absorption λ_{max} (about 340 nm). Piperine was found to bind to HSA (Fig. 9A) but on exposure to UVA (Fig. 9B) or SSR (Fig. 9C), this was abolished, suggesting that the structural changes induced by radiation were detrimental to protein binding of the molecule. No binding to DNA was observed by CD either before or after irradiation (Fig. 10A–C); the increase in absorbance at 275nm (Fig. 10B,C) is due to a concentration effect (solvent evaporation). Absence of binding to DNA was confirmed with the use of linear dichroism (data not shown). This suggests that unlike the situation with psoralens (13), piperine and its isomers do not bind DNA and would not form photoadducts *in vivo*.

The present results show that UVR-induced photoisomerization of the piperine molecule results in the loss of its protein binding and melanocyte stimulatory activity. These results do not, however, preclude the use of UVR in conjunction with piperine in the treatment of vitiligo. We have conducted *in vivo* experiments with a hairless, sparsely pigmented mouse strain (HRA.HRII-c+/Skh) in which piperine solution was applied topically twice every weekday for 9 weeks and UV irradiation was administered three times a week from Weeks 5–9, just prior to application of piperine. Using this protocol, the melanocyte stimulatory effect of piperine was retained. Pigmentation was better in mice receiving both piperine and UVR than in mice treated with either agent alone (Fig. 11), verifying the usefulness of their concomitant use. It is important that administration of UV irradiation to patients receiving piperine must be appropriately timed and that piperine-containing preparations are protected from light.

CONCLUSIONS

When exposed to physiologically relevant doses of UVA and SSR, piperine photoisomerizes at the conjugated double bond to give a mixture of isomeric products. UV and ^{1}H NMR data provided good evidence for the presence of chavicine and isochavicine in the mixture, although the presence of isopiperine could not be ruled out. Piperinic acid, a potential hydrolysis product of piperine, was not detected. Conversion to its geometric isomers led to a loss of piperine's ability to stimulate melanocyte proliferation and to bind to HSA *in vitro*. No *in vitro* binding to DNA was observed either before or after irradiation. These results are not surprising, given

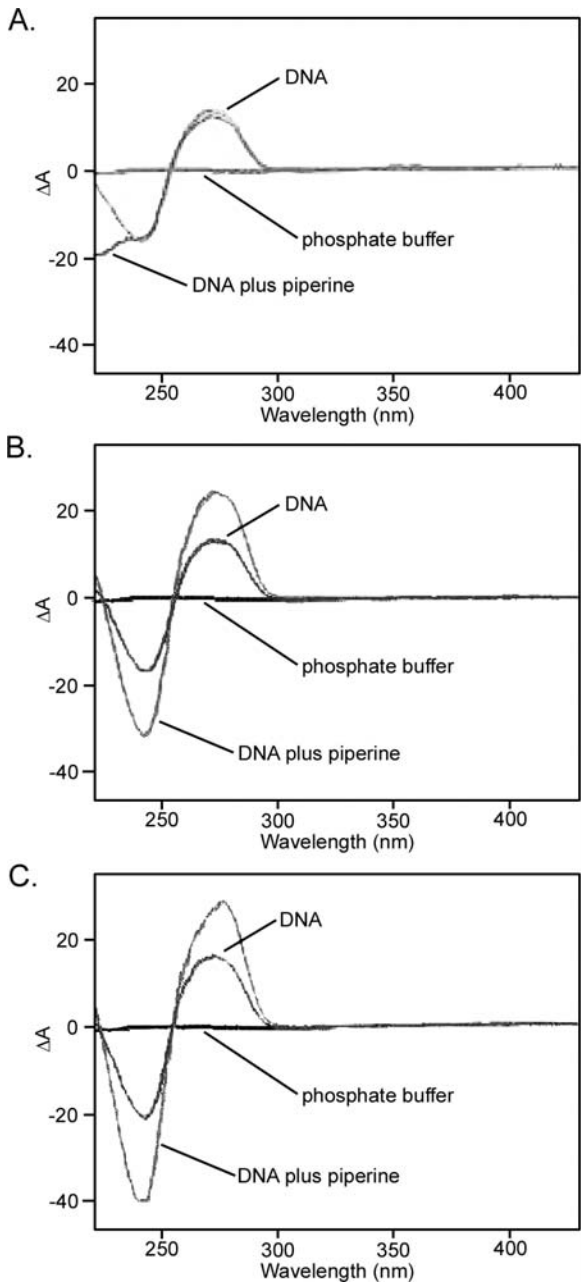


Figure 10. CD study of piperine's interaction with DNA, before exposure to irradiation (A), after exposure to UVA (22 J cm^{-2}) (B), and after exposure to SSR (15 J cm^{-2}) (C).

that piperine absorbs UVR across the whole solar range (Fig. 2). Thus both UVB and UVA are likely to have contributed to the photoisomerization and loss of melanocyte stimulatory activity observed here with environmentally and physiologically relevant doses of SSR. If UVR is used with piperine in the treatment of vitiligo, application of the compound and irradiation should take place at different times, to minimize photoisomerization of piperine. This protocol was more effective than piperine or radiation alone in inducing pigmentation in a sparsely pigmented mouse strain. Bright sunlight should be avoided both during active application of piperine to the skin and in the storage of piperine products.

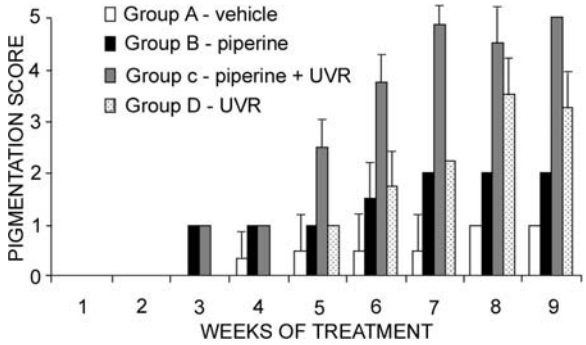


Figure 11. Pigmentation scores in mice ($n = 4$ per group) treated with (A) topical DMSO (vehicle) for 9 weeks, (B) topical piperine in DMSO for 9 weeks, (C) topical piperine in DMSO for 9 weeks with UVR from Weeks 5–9, and (D) UVR alone from Weeks 5–9. Piperine treatment (B) gave significantly ($P < 0.05$) higher scores than vehicle alone (A). Treatment with piperine and UVR (C) gave significantly ($P < 0.05$) better pigmentation scores than either piperine alone (B) or UVR alone (D). Groups were compared with the use of the Mann–Whitney *U*-test.

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