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In vivo evaluation of piperine and synthetic analogues as potential treatments for vitiligo using a sparsely pigmented mouse model

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Conflicts of interest

None declared.

Summary

Background Piperine and its analogues have been reported to stimulate melanocyte replication *in vitro* and may be useful in treating the depigmenting disease, vitiligo.

Objective To investigate the ability of piperine (PIP) and three analogues to stimulate pigmentation in a strain of sparsely pigmented mice.

Methods The test compounds were PIP [5-(3,4-methylenedioxyphenyl)-2,4-pentadienoylpiperidine], tetrahydropiperine [THP, 5-(3,4-methylenedioxyphenyl)-pentanoylpiperidine], a cyclohexyl analogue of piperine [CHP, 5-(3,4-methylenedioxyphenyl)-2,4-pentadienoylcyclohexylamine], and reduced CHP [rCHP, 5-(3,4-methylenedioxyphenyl)-2,4-pentanoylcyclohexylamine]. Sparsely pigmented, HRA/Skh-II mice were randomized to receive topical treatment with test compounds or vehicle twice a day for five days a week, with or without ultraviolet (UV) irradiation on 3 days a week. Treatment was either continuous or interrupted to evaluate fading and repigmentation. Skin inflammation and pigmentation were evaluated regularly during treatment. DOPA+ melanocytes were determined histologically at the termination of treatment.

Results Four weeks of treatment with one of the compounds PIP, THP or rCHP, but not CHP, induced greater pigmentation than vehicle with low levels of inflammation. Additional exposure to UVR led to darker pigmentation than did the compound or UVR alone, and greater numbers of DOPA+ melanocytes were found. The combination produced an even pigmentation pattern, contrasting with the speckled, perifollicular pattern produced by UVR alone. Treatment interruption led to a decrease in pigmentation but not its loss. Repigmentation was achieved by administering one of the compounds, UVR or both, and occurred faster than in naïve mice.

Conclusions Treatment with PIP, THP or rCHP and UVR induced a marked pigmentation response in HRA/Skh-II mice, with clinically better results than UVR alone. This result supports the potential use of these compounds in treating vitiligo.

The skin disorder vitiligo is the most common acquired hypomelanosis, affecting approximately 1% of the world's population, with serious cosmetic and psychological effects.[1] The characteristic depigmentation can be restricted to a limited skin area (segmental vitiligo) or generalized in symmetrical patches (nonsegmental vitiligo). In most cases, loss of skin colour corresponds with melanocyte loss, first in the epidermal compartment, and later in the follicular reservoir where most melanocytic stem cells are probably situated.[2]

Treatment of vitiligo is often difficult and disappointing. This is most probably because the aetiopathogenesis is unknown, and a treatment directed to the cause has not

been established. Several treatment modalities, such as PUVA [psoralen + UVA (320–400 nm) radiation], broad-band (280–320 nm) and narrow-band (311 nm) UVB, and local corticosteroids are currently used. However, it has been reported that these standard treatments result in limited success; less than 25% of patients responded successfully to topical corticosteroids.[3] Moreover, corticosteroids applied either systemically or topically carry the risk of significant side effects in long-term therapy.[4] Alternatively, PUVA therapy seldom achieves extensive repigmentation that is cosmetically acceptable, and treatment response is often followed by relapse.[5] A recent Cochrane review[6] highlights the lack of research on current treatments as well as the need to identify novel clinical approaches for vitiligo.

Several clinical studies[7–10] strongly suggest that reservoirs in hair follicles are the source of melanocytes in skin repigmented by standard therapies. Small circular areas of repigmentation centred around hair follicles enlarge and eventually coalesce. Consequently, the identification of stimuli that activate outer root sheath melanocytes is a prospective means of developing new treatments for vitiligo.

Recent evidence from our laboratory indicates that piperine [5-(3,4-methylenedioxyphenyl)-2,4-pentadienoylpiperidine; PIP] has a potent stimulatory effect on mouse melanocytes *in vitro*. Culture media supplemented with *Piper nigrum* (black pepper) fruit extract or its main alkaloid, PIP, induced nearly 300% stimulation of melan-a mouse melanocyte proliferation after 8 days of treatment *in vitro*.[11] The increase of growth was effectively inhibited by RO-31-8220, a broad-spectrum protein-kinase C (PKC) inhibitor, suggesting that PKC signalling is involved in its activity. Both *Piper nigrum* extract and PIP also induced an increase in the number and length of cell dendrites.[11] Melanin synthesis, however, was not stimulated. We have also shown that several synthetic derivatives of piperine share these *in vitro* effects.[12]

The aim of the present study was to evaluate the melanocyte-stimulatory activity of PIP and three of its synthetic derivatives (Fig. 1) *in vivo* as a putative new chemical group for the treatment of vitiligo, alone or in association with UVR. Studies were performed in HRA.HRII-c/+/Skh mice, a hairless, sparsely-pigmented mouse model[13] that has white skin except for the ears and tails. 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, Philadelphia, PA, U.S.A. These mice have melanocytes in the epidermal layer (as in human skin), whereas in many other pigmented mouse strains, melanocytes are found only in the dermis. The numbers of epidermal melanocytes in this model are small – two or three DOPA+ melanocytes mm^{-2}.[14] However, unlike albino mice, pigmentation in HRA.HRII-c/+/Skh mice is inducible[13,14] with melanocyte numbers reaching close to 600 mm^{-2}. As with vitiligo, perifollicular pigmentation is evident after exposure to UVR with and without photosensitizers.[13–15] We therefore advocate the induction of pigmentation in this strain as an *in vivo* model for repigmentation in vitiligo.

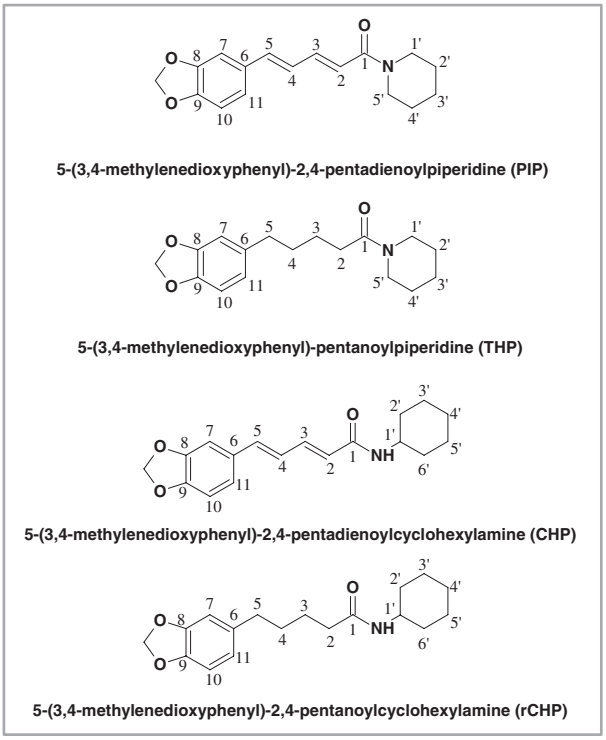

Fig 1. Piperine (PIP) and structural analogues tetrahydropiperine (THP), a cyclohexyl analogue of piperine (CHP) and reduced CHP (rCHP).

Materials and methods

Animals

Male and female inbred HRA.HRII-c/+/Skh hairless pigmented mice, age-matched (8–16 weeks old), were used. Animals were bred by the Biological Services Division, KCL, University of London, U.K. and the Rayne Institute, St Thomas's Hospital, London, U.K. Animals were killed by cervical dislocation and skin samples removed surgically when required.

Chemicals

PIP [5-(3,4-methylenedioxyphenyl)-2,4-pentadienoylpiperidine] was purchased from Sigma-Aldrich Ltd (Dorset, U.K.). PIP derivatives, i.e. tetrahydropiperine [5-(3,4-methylenedioxyphenyl)-pentanoylpiperidine; THP], a cyclohexyl analogue of piperine [5-(3,4-methylenedioxyphenyl)-2,4-pentadienoylcyclohexylamine; CHP] and reduced CHP [5-(3,4-methylenedioxyphenyl)-2,4-pentanoylcyclohexylamine; rCHP] were synthesised in our laboratory.[12]

Selection of vehicles by *ex vivo* skin assays

To determine the optimum vehicle for delivery of test agents, *ex vivo* permeation studies were conducted with vertical Franz diffusion cells using a modification of reported methods.[16]

Each cell consists of a chamber with upper (donor) and lower (receiver) compartments divided by the mounted skin sample. The skin acts as a seal between the two half-cells when they are clamped together. The upper, stratum corneum side is filled with the drug formulation and the lower one (dermal side) with receiving fluid. Samples of the receiving fluid are taken at intervals to quantify the amount of the drug passing through the skin. The cells used in this study had a 10 mL capacity receptor compartment and a 1·75 cm^2 diffusion area. A circular piece of full-thickness dorsal skin from HRA.HRII-c+/Skh mice was carefully mounted onto the receiver compartment of the diffusion cells with the stratum corneum facing the donor compartment. The receptor compartment was filled with phosphate buffered saline; PBS, pH 7·4) which was continuously stirred with a magnetic bar. Test solutions [175 mmol L^{-1} PIP in ethanol, diethylene glycol monoethyl ether (Transcutol®, Gattefossé, Saint-Priest Cedex, France), dimethyl sulfoxide (DMSO), polyethylene glycol (PEG) or 5% oleic acid (OA) in PEG] were added into the donor compartment of each cell ($n = 4$ for each formulation). Samples of fluid from the receiver cell were taken at 3, 19 and 22 h and the concentration of PIP was determined by high performance liquid chromatography (HPLC) using a model 3100 pump (LDC Analytical, Riviera Beach, FL, U.S.A.) with a Spectromonitor 3100 UV detector (LDC Analytical) and Hewlett Packard 3390 A integrator. A 4·6 × 25 cm, 10 μm, C18 Econosil reverse phased column (Alltech U.K., Stamford, U.K.) was used, eluting with methanol : water (60 : 40; HPLC grade, 1 mL min^{-1}). The detector wavelength was set at 348 nm. Under these conditions, PIP eluted at 10·59 min. Results were expressed as mg mL^{-1} according to a previously determined calibration curve (0·003–0·1 mg mL^{-1} PIP in PBS).

Topical application of test compounds *in vivo*

Test agents were dissolved in vehicle (either OA/PEG or in DMSO) to a final concentration of 175 mmol L^{-1} and 100 μL (17·5 μmoles) applied with a micropipette on the central area of mouse dorsal skin (2–3 cm^2), twice a day (weekdays only) with an interval of 5–6 h between applications. In protocols with UVR exposure, the irradiations were carried out every Monday, Wednesday and Friday immediately prior to the first daily application, to avoid a possible photosensitizing effect and/or photodamage[17] to the test compound.

UV irradiation and dosimetry

The UVR source was a bank of eight Bellarium SA-1-12-100W fluorescent tubes (Wolff, Erlangen, Germany), the emission spectrum of which has been published.[13] This UVR source emits 4·1% UVB (280–320 nm) and 95·8% UVA (320–400 nm), but the UVB accounts for the 71·5% erythemally effective energy when biologically weighted with the human erythema spectrum.[18] Given that tanning and erythema action spectra are very similar[19] it is probable that the small UVB component accounts for most of the tanning effect. Irradiations were carried out in a purpose-built unit with ventilation, temperature and humidity controls. The irradiance was monitored daily immediately before irradiation with an International Light radiometer (IL 422A; Newburyport, MA, U.S.A.) equipped with UVR sensors. The radiometer was calibrated for the source, as described before.[13] Irradiance measured at mouse level was typically about 0·16 mW cm^{-2}. Animals were irradiated unrestrained in metal cages with a dose of 354 mJ cm^{-2},[13] confirmed to be sub-inflammatory from a single exposure (increase in skin fold thickness (SFT) < 10%; data not shown). Irradiations lasted for a maximum of 1 h. The position of cages was systematically rotated to ensure even UVR exposure.

Experimental groups

In initial experiments, animals were treated topically with PIP, THP and CHP dissolved in either OA/PEG or in DMSO or with vehicle alone for 9 weeks with concomitant exposure to UVR during weeks 5–9. Further experiments (summarized in Fig. 2) were conducted using compounds dissolved in DMSO, with DMSO as control. For continuous treatment, animals ($n \geq 4$) were treated topically with PIP, THP, rCHP or DMSO for up to 13 weeks (Fig. 2, Group A). A second group (Fig. 2, Group B) received the same treatment, but was additionally exposed to UVR from week 5 to 13. For studies on discontinuous treatment, animals were treated as in Group B up to week 7. All treatment was then suspended for 3 weeks (weeks 8–10) and re-started as topical application only (Fig. 2, Group C), UVR only (Fig. 2, Group D), or topical application plus UVR (Fig. 2, Group E) for weeks 11–13. Mice exposed only to UVR (i.e. no vehicle treatment) from week 5 onwards (Fig. 2, Group F) were used as controls for all groups treated with UVR.

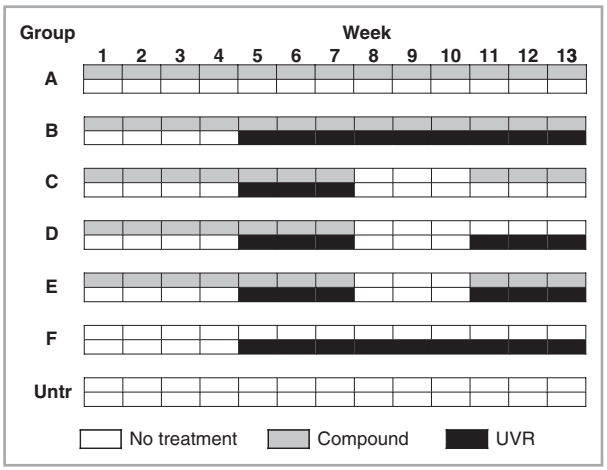

Fig 2. Treatments. Mice were treated for 13 weeks with topical compounds alone (A) or with additional ultraviolet radiation (UVR) exposure (B). For other groups, treatment was interrupted and restarted as compounds only (C), UVR only (D), or compounds + UVR (E). Group F received UVR alone.

Assessment of inflammation and pigmentation

Dorsal SFT was recorded to evaluate potential inflammatory effects of treatments, Measurements were taken every day during the first week of treatment and twice a week thereafter with a spring-loaded micrometer (Mitutoyo, Kawasaki, Japan). Pigmentation was assessed independently by two investigators and the average score calculated. The first type of assessment was conducted visually, every day, and pigmentation scored from 0 to 5 according to the following scheme: 0 = no pigmentation; 1 = first signs of pigmentation (freckles); 2 = light brown; 3 = medium brown; 4 = dark brown; 5 = black. Pigmentation was also assessed histologically by DOPA staining at the end of the experiment. Animals were killed and skin samples from representative dorsal areas (1 cm^2) were removed surgically and incubated in 2 mol L^{-1} NaBr in PBS for 2 h at 37 °C. The epidermis was carefully removed with tweezers and further incubated in 0·1% L-DOPA in PBS (pH 7·2) for 4 h at 37 °C. The DOPA solution was changed periodically to prevent auto-oxidation. Finally, epidermal sheets were fixed in 4% paraformaldehyde in PBS (pH 7·4) for 15 min, dehydrated through a graded series of alcohol concentrations and mounted on glass microscope slides for examination. The number of DOPA+ cells per mm^2 was calculated from at least 30 fields per sample (n = 4 animals). DOPA+ cells were also classified as highly or poorly melanized according to their melanin granule content. The percentage of cells in each category per mm^2 was calculated.

Results

Selection of vehicles based on skin penetration measured with Franz cells

Skin penetration of PIP when dissolved in five vehicles was compared using Franz cells. At 22 h after application, the concentration of PIP in the receiver compartment was highest with DMSO followed by OA/PEG in both male and female skin (Fig. 3). PIP was undetectable when delivered in other vehicles (ethanol, diethylene glycol monoethyl ether and PEG). No PIP was detected at shorter time periods (3 h and 19 h) with any vehicle. DMSO and OA/PEG were therefore chosen as vehicles for the *in vivo* studies.

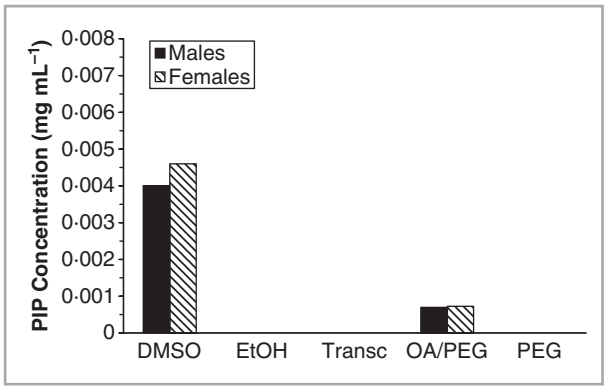

Fig 3. DMSO is the best of five skin penetration enhancers for piperine (PIP). Penetrance of PIP dissolved in ethanol (EtOH), DMSO, diethylene glycol monoethyl ether (Transc), polyethylene glycol (PEG) and 5% oleic acid in PEG (OA/PEG) through HRA.HRII-c+/Skh mouse dorsal skin was tested using Franz cells.

Inflammatory and irritant effects

Differences in inflammatory response were seen depending on the vehicle, test compound and sex of animal. OA/PEG based formulations induced stronger adverse effects than those with DMSO in both male and female mice (Figs 4 and 5). However, in males, PIP and THP solutions in OA/PEG induced a stronger inflammatory response (more than 30% increase in SFT; Fig. 4a), than in females where THP had only a mild inflammatory effect (20% increase in SFT, Fig. 5a). The inflammatory effect of CHP was comparable to vehicle alone in both males and females (around a 20% increase in SFT, Figs 4a and 5a). The inflammatory response induced by formulations

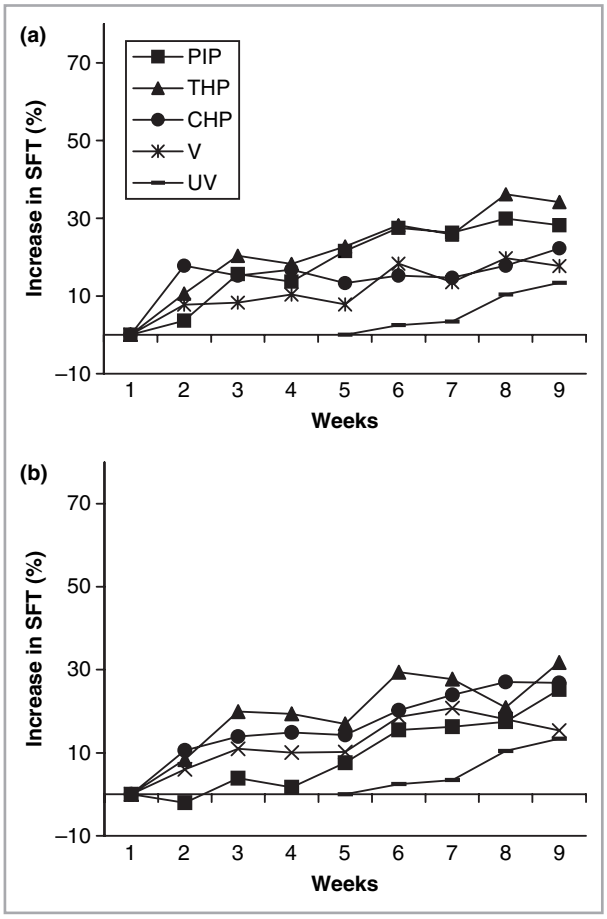

Fig 4. Inflammation in male mice treated with piperine (PIP), tetrahydropiperine (THP) or cyclohexyl analogue of piperine (CHP) in 5% oleic acid in polyethylene glycol (OA/PEG) (a) or DMSO (b). Inflammation was assessed as percentage increase in skin fold thickness (SFT). Only mean values (n = 5) are given for clarity (% CV not more than 30%). V, vehicle; UV, ultraviolet radiation.

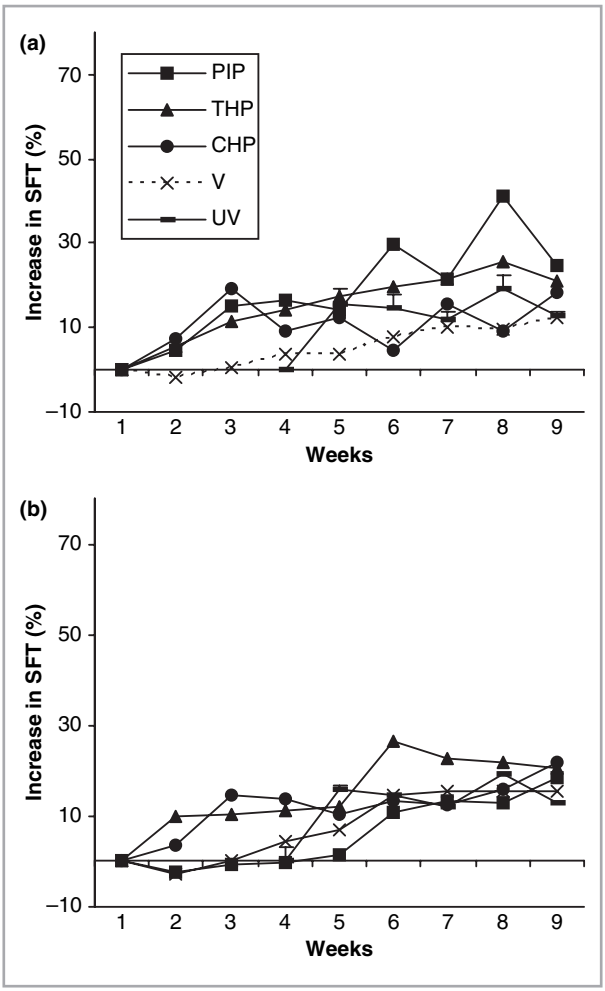

Fig 5. Inflammation in female mice treated with piperine (PIP), tetrahydropiperine (THP) and cyclohexyl analogue of piperine (CHP) in 5% oleic acid in polyethylene glycol (OA/PEG) (a) or DMSO (b). Inflammation was assessed as percentage increase in skin fold thickness (SFT). Only mean values ($n = 5$) are given for clarity (% CV not more than 20%). V, vehicle; UV, ultraviolet radiation.

in DMSO was observed to be milder than with OA/PEG. DMSO alone had a mild inflammatory effect (up to 20% increase in SFT; Figs 4b and 5b). Both vehicles and test solutions had a transient irritant effect, producing redness and desquamation of treated areas during the first 10 days of treatment (data not shown). Irritancy induced by DMSO (and DMSO-based solutions) was milder than with OA/PEG. Redness decreased after an hour of topical application, and desquamation was less severe than with OA/PEG. PIP and THP in OA/PEG showed the most powerful irritant effects, in agreement with the strong inflammatory response observed. The irritant effects were overcome by week 2 of topical treatment. UVR alone had lower inflammatory effects than any of the topical treatments.

Pigmentation

Four weeks of topical treatment with PIP or THP, in either DMSO (Fig. 6a) or OA/PEG (not shown), induced a light, even pigmentation of the treated area compared with vehicle control whereas CHP had virtually no effect. The vehicles used also showed some effect, as previously reported for DMSO[20,21] and OA.[22–24] For PIP (Fig. 6b) and THP, subsequent suberythemal exposure to two UVR exposures alone significantly enhanced pigmentation induced by the test compounds compared with controls treated with vehicle and UVR, or UVR alone. Pigmentation was observed as a dark, even pattern after 6–8 exposures (Fig. 6c,d). The pigmentation induced by topical treatment with vehicle was lighter and uneven (DMSO, Fig. 6a–d). The pigmentation induced by UVR alone (Fig. 6c) was observed to be perifollicular and therefore speckled, in contrast to the even pigmentation of PIP and THP alone (Fig. 6a), or in combination with UVR (Fig. 6b–d).

Different pigmentation responses observed *in vivo* corresponded with changes in the number of DOPA+ cells mm^{-2} in the skin (Fig. 7). Pigmentation responses were slower and less evident in females (scores not shown) than in males. In male mice, treatment with PIP and THP in either DMSO or OA/PEG significantly ($P < 0{\cdot}05$) increased the number of DOPA+ cells compared with vehicles. The lower pigmentation responses in female mice corresponded with a smaller mean number of DOPA+ cells mm^{-2} under all treatment conditions compared with males receiving equivalent treatments (Fig. 7). In females the stimulatory effects of THP and PIP on pigmentation reached statistical significance only with PIP in OA/PEG and THP in DMSO (Fig. 7) although a trend towards an increase was apparent with both compounds in either vehicle. CHP, in contrast, did not show any effect on the number of DOPA+ cells compared with vehicles in either males and females, in agreement with the low pigmentation levels observed on visual examination of the animals (Fig. 6).

Further experiments were carried out in order to determine the persistence of the pigmentation effect after the cessation of treatment and the stimuli needed to restore pigmentation if lost, according to protocols summarized in Fig. 2. PIP, THP and a novel compound, the reduced form of CHP (rCHP) were tested. rCHP was chosen because of its high stimulatory activity on melanocyte proliferation *in vitro*.[12] All solutions were prepared in DMSO because of its better performance as a penetration enhancer (Fig. 3) and milder inflammatory effects than OA/PEG (Figs 4 and 5). The results of this experiment are shown in Fig. 8. The application of the compounds alone for 13 weeks (Group A) stimulated pigmentation up to a maximum of level 2 (light brown). The first change was observed at 4 weeks and maximum pigmentation was reached by week 6. The application of concomitant UVR (Group B) significantly enhanced pigmentation reaching up to level 5 (black) by week 7. This was greater than the highest mean scores obtained with either compound alone (Group A; score 2, light brown) or UVR alone (Group F; score 3, medium brown). In mice treated topically with a compound, four exposures of suberythemal doses of UVR were sufficient to induce a pigmentation score of 3 (Group B), in contrast with the greater number of UVR exposures (more than 10) needed

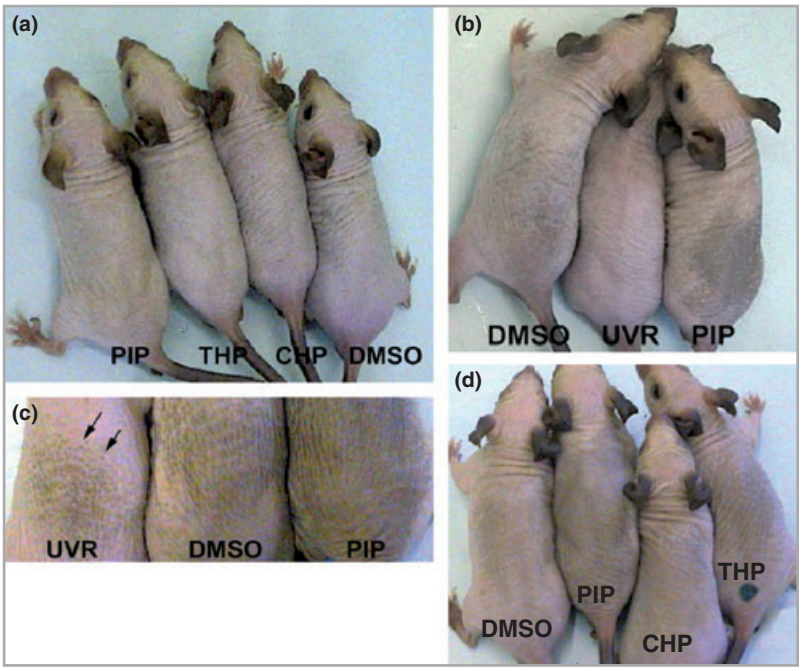

Fig 6. Pigmentation induced in male mice by piperine (PIP) and derivatives applied in DMSO. (a) PIP and tetrahydropiperine (THP) [but not cyclohexyl analogue of piperine (CHP)] applied for 4 weeks induce greater pigmentation than DMSO. After two ultraviolet radiation (UVR) exposures (b) or eight UVR exposures (c,d), pigmentation is darker in mice treated with PIP or THP (but not CHP) than in mice treated with DMSO or previously untreated (UVR) mice. Compounds produce an even pigmentation compared with the speckles (c, arrows) caused by UVR alone.

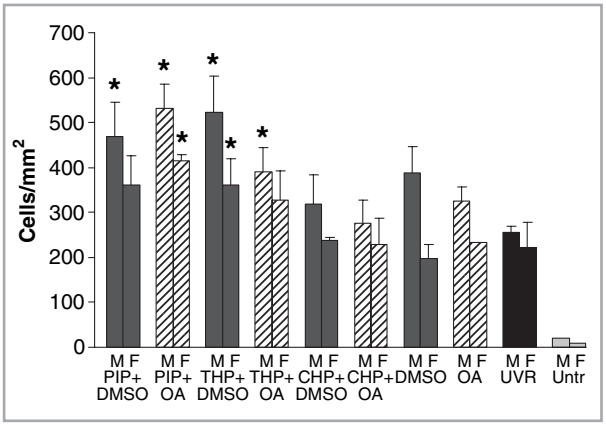

Fig 7. Increase of DOPA+ cell number after continuous treatment with piperine (PIP), tetrahydropiperine (THP) or cyclohexyl analogue of piperine (CHP) in DMSO or 5% oleic acid in polyethylene glycol (OA/PEG) for 9 weeks, with exposure to ultraviolet radiation (UVR) from week 5 to 9 (mean ± SD; n = 4). (M), male; (F), female. *P < 0·05 compared with vehicle (Student's t-test). Untr, untreated (naïve).

to obtain a similar, but less even, response in naïve mice (Group F). This data clearly shows a combined pigmentation enhancing effect of PIP, THP and rCHP with UVR. Pigmentation in Group B was maintained up to week 13 with continued treatment with the compounds plus UVR.

After three weeks without treatment (Week 8–10), the degree of pigmentation decreased in animals treated with a compound and UVR (Fig. 8, Groups C, D and E) compared with week 7 pigmentation levels, but did not disappear completely. By contrast, there was no remaining detectable pigmentation after week 9 in animals treated with only DMSO and UVR in both male (Fig. 8, Group C, D and E) and female (data not shown) mice. Retreatment with topical solutions, UVR, or a combination of both, all resulted in re-pigmentation after 3 weeks (Week 11–13; Fig. 8, Group C, D and E). The rate of increase in pigmentation was faster than the initial pigmentary response (weeks 1–4), reaching scores of 2 or more within 2 weeks of retreatment (Week 12; Group C, D and E). Retreatment with the compounds alone (Fig. 8, Group C) increased pigmentation to levels comparable with those obtained by continuous topical treatment alone (Fig. 8, Group A). Retreatment with UVR alone (Fig. 8, Group D) or combined topical applications plus UVR (Fig. 8, Group E) resulted in higher pigmentation levels (score 3) than after retreatment with the compounds alone (score about 2), but comparable with UVR alone (Fig. 8, Group F). The pigmentation patterns resulting from a compound alone (Group C) or a compound plus UVR (Group E) were both even (Fig 6c and data not shown). In contrast, pigmentation induced by retreatment with UVR (Fig. 8, Group D) resembled the spotted pattern obtained with continuous UVR exposure (Fig. 6c).

Histological analysis of skin melanocyte numbers (Fig. 9) again showed a good correlation with visually observable differences in pigmentation for both male and female mice. Group B animals (compound plus UVR) showed significantly more melanocytes mm^{-2} than those receiving compounds (Group A) or UVR (Group F) alone. Based on the fading of pigmentation, treatment withdrawal (Groups C–E, weeks 8–10) is assumed to have caused a decrease in the activity of melanocytes. In males (Fig. 9a), retreatment with UVR alone (Group D) or with topical compounds plus UVR (Group E) increased the number of DOPA+ cells mm^{-2} at the end of Week 13 to levels comparable with those in Group B which

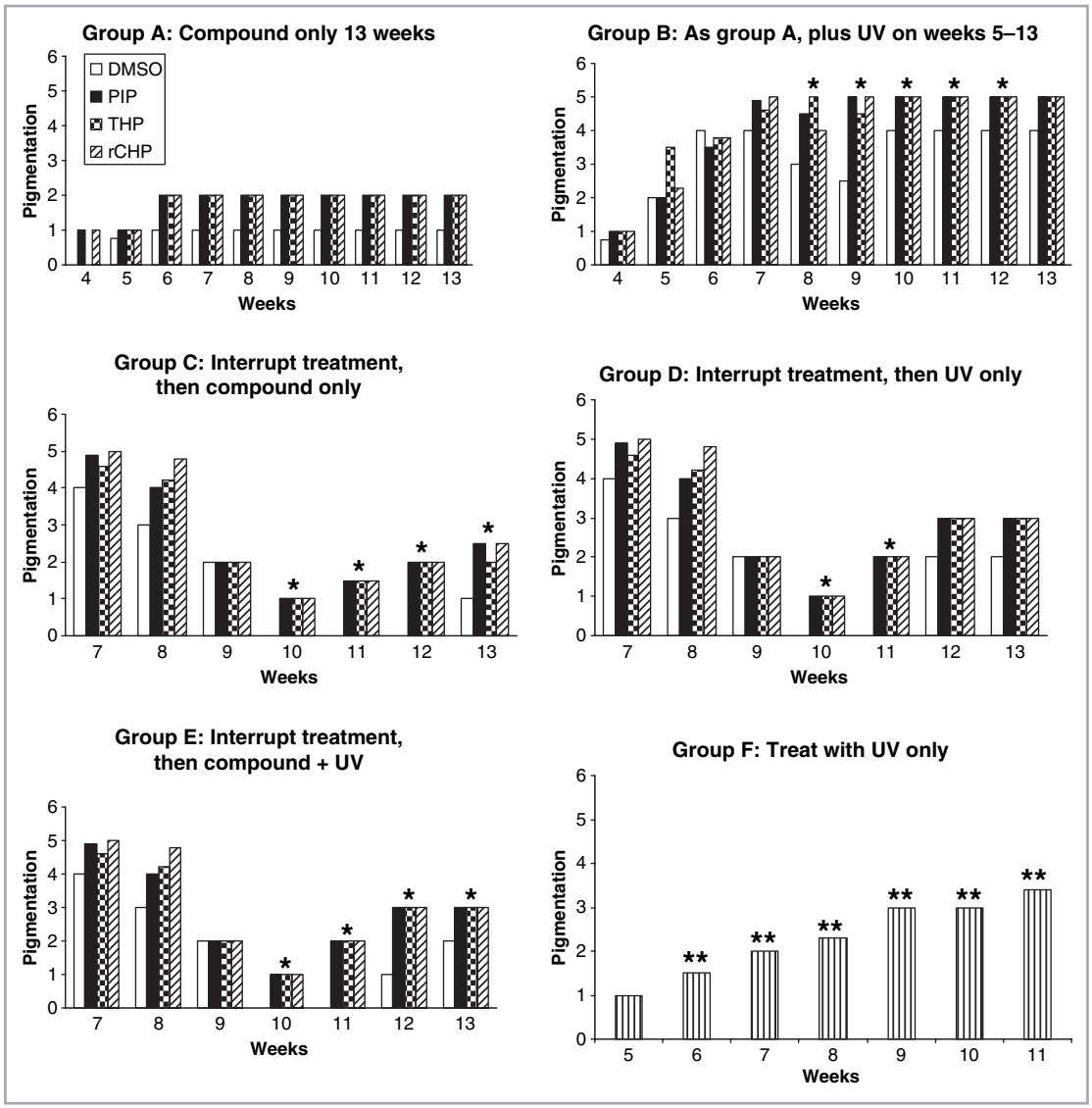

Fig 8. Pigmentation response of male mouse skin (n = 4) to continuous (Groups A, B, F) or discontinuous (Groups C–E) treatment as summarized in Figure 2. Mice were treated for 13 weeks with topical compounds alone (A) or with additional ultraviolet radiation (UVR) exposure (B). For other groups, treatment was interrupted and restarted as compounds only (C), UVR only (D), or compounds + UVR (E). Group F received UVR alone. Pigmentation scores range from 1 (freckles) to 5 (black). *$P < 0{\cdot}05$ compared with vehicle; **$P < 0{\cdot}05$ compared with Group B (Mann–Whitney U-test).

had received continuous treatment with UVR and a compound. The number of cells mm^{-2} in Group D animals was significantly higher than in the group that received continuous UVR (Group F) or continuous topical treatment (Group A). Retreatment with a compound alone significantly increased the number of DOPA+ cells mm^{-2} compared with vehicle control (Group C), reaching comparable levels with those in animals treated continuously with a compound alone (Group A). However, the number of cells mm^{-2} was lower than for groups that were retreated with UVR alone (Group D) or with a compound and UVR (Group E). The results obtained in female mice (Fig. 9b) showed the same trends as in males, except that in animals retreated with UVR alone (Group D) or UVR with a compound (Group E) no significant differences were observed compared with vehicle controls.

To investigate whether the differences in pigmentation observed were due to an increase in melanocyte number or in melanin production, DOPA+ cells were classified as highly or poorly melanized according to the content of pigment granules, and the percentage of DOPA+ cells in each category per mm^2 of skin was calculated for each experimental group. As expected, UVR exposure considerably increased the degree of melanization of DOPA+ cells (Fig. 10c and Groups B, D, E and F in Fig. 11a,b) compared with mice treated with a compound alone (Fig. 10b or Groups A and C in Fig. 11a,b), where poorly melanized cells were predominant.

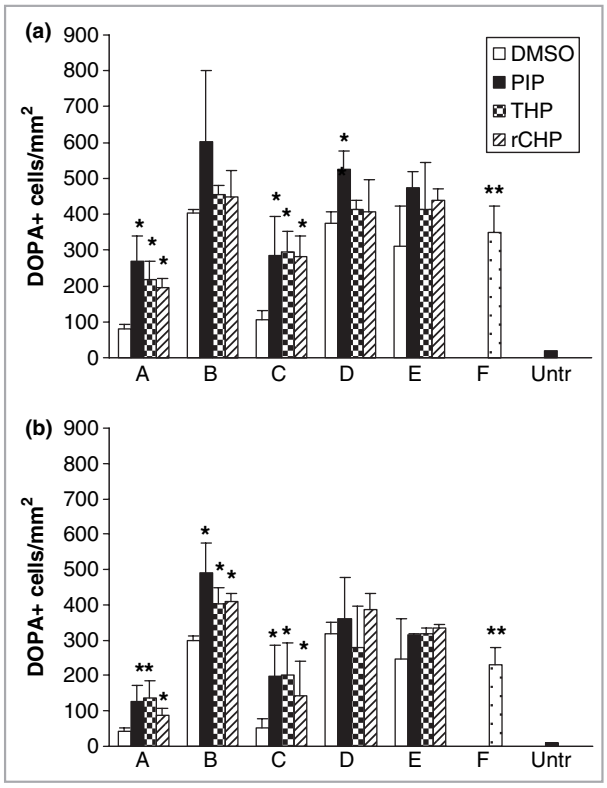

Fig 9. DOPA+ cell numbers (mean ± SD) in male (a) and female (b) mouse skin ($n = 4$) after continuous and discontinuous treatment as in Fig. 2, Groups A–F). Cell numbers correlate well with visually determined pigmentation scores. *Significant increase compared with vehicle; **Significant decrease compared with groups B and D ($P < 0{\cdot}05$; Student's t-test).

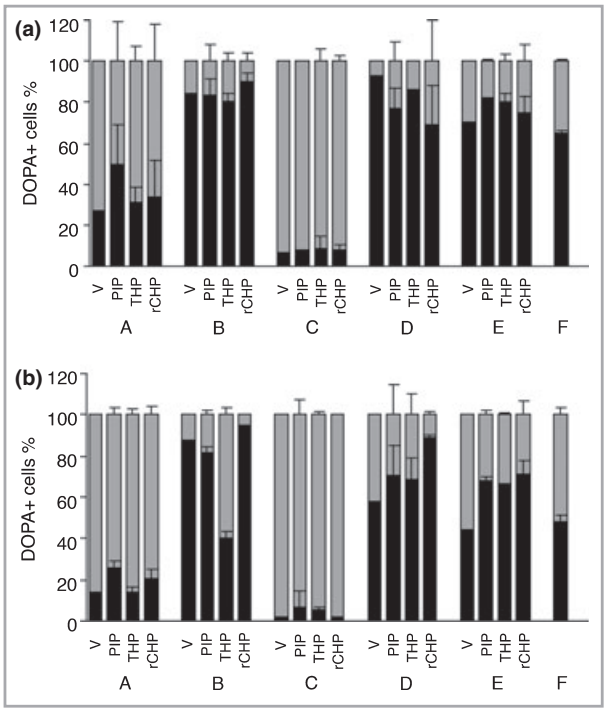

Fig 11. Percentage of highly (black bars) vs. poorly (grey bars) melanized melanocytes in male (a) and female (b) mice (mean ± SD; $n = 4$). Groups receiving ultraviolet radiation (UVR) (B, D, E and F) show greater melanization than those receiving a compound alone (A, C) prior to histology. Treatment groups as shown in Figure 2.

Discussion

Topical treatment of HRA/Skh-II mice with PIP, or two of its synthetic derivatives, THP and rCHP, stimulates the development of even skin pigmentation *in vivo* after four or more weeks of continuous topical application. The darkening of skin in treated areas corresponds with an increase in the number of DOPA+ melanocytes. This *in vivo* finding correlates well with our previous studies showing the stimulation of *in vitro* melanocyte proliferation by PIP and chemically related compounds.[11,12] Animals treated with PIP or analogues before UVR exposure showed more rapid and darker pigmentation than those treated with UVR exposure or a compound alone (Fig. 8). These findings highlight the potential of these compounds as novel treatments for vitiligo. Notably, supplementing UVR with these compounds may offer a means of reducing UV exposure in vitiligo therapy, thereby reducing the risk of developing skin cancer.

The degree of skin pigmentation is a consequence of both number of melanocytes and their degree of melanization. UVR, for example, stimulates both melanocyte proliferation

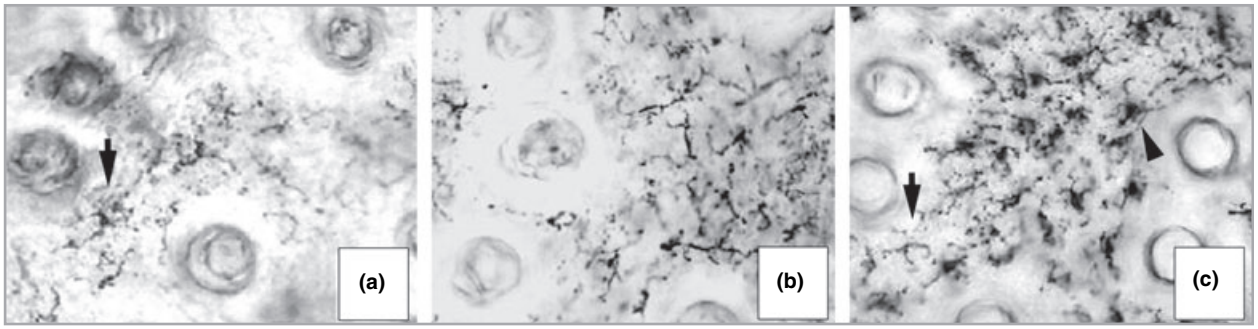

Fig 10. Histology of DOPA+ melanocytes in skin from animals treated with (a) vehicle, (b) piperine (PIP) and (c) PIP + ultraviolet radiation (UVR). The ratio of highly (arrowheads) to poorly (arrows) pigmented melanocytes increases in the order a < b < c. Original magnification × 200.

and melanin synthesis.[21,25] The relatively low pigmentation scores in the absence of UVR (Fig. 8) and the low degree of melanization of DOPA+ cells observed in skin treated with a compound alone (Fig. 11) suggests that these compounds stimulate melanocyte proliferation rather than melanin synthesis. This is in good agreement with *in vitro* data showing that PIP derivatives do not stimulate melanin production although they stimulate melanocyte proliferation.[11,12] Retreatment with a compound alone induced a higher difference in DOPA+ cell numbers between compound and vehicle (Fig. 9a,b, Group C) than did retreatment with UVR alone (Fig. 9a,b, Group D), This again suggests that the primary effect of piperine is to stimulate rapid melanocyte proliferation and population of epidermal areas. This phenomenon, as well as effects on melanocyte differentiation by PIP analogues could be further examined through bromodeoxyuridine incorporation experiments and immunohistochemical determination of specific markers such as Kit, Mitf, TRP-1 and TRP-2, indicative of different developmental stages of melanocytes.[26]

A gender difference in induced pigmentation was observed in these studies, with males showing a greater response than female mice. However, skin penetration of PIP was the same in both sexes using a Franz cell model (Fig. 3), suggesting equal bioavailability in both sexes. However, the mild inflammatory and irritant effects seen with PIP and its analogues (Figs 4 and 5) may be significant, in explaining the activity of the compounds *per se*, as well as the differences in pigmentary response of male and female animals. Females showed a lower inflammatory response than males. Gender differences in sensitivity to UVR have also been observed in humans with males showing a greater sensitivity and lower MED.[27]

An important feature of treatment with PIP and its analogues is the even pigmentation pattern that is obtained with or without additional UVR (Fig. 6). This correlates well with the finding that DOPA+ melanocytes in treated skin (Fig. 10) were distributed in interfollicular areas rather than associated with hair follicles, and suggests an active epidermal distribution of melanocytes after treatment with PIP or its analogues. An examination of the *in vivo* cutaneous absorption and distribution of PIP and its analogues, particularly the relative roles of the stratum corneum and hair follicles, would be of interest in determining their site of action and understanding the repigmentation patterns seen. Hair follicles are known to play a significant role in the percutaneous absorption of many drugs.[28] The use of PIP and its analogues in vitiligo clearly offers potential cosmetic advantages over the use of PUVA or UVR alone (common current treatments for vitiligo) if an even pattern can be obtained in humans. PUVA repigmentation, when successful, progresses from a perifollicular pattern in early stages of therapy, with the circular patches of pigment coalescing after further treatment to a more even pattern in humans[5] and in mice.[15] A similar progression has been observed using therapies based on UVR.[9,29] Mice treated with UVR alone in the present study also showed this speckled pattern (Fig. 6c).

Continuous treatment appears to be needed to maintain pigmentation as shown by the gradual, though not complete, loss of pigmentation when treatment is suspended. Retreatment with either UVR alone, topical compounds alone or the combination of both, restored pigmentation over a shorter period of time than in naïve mice. This indicates the possible presence of poorly melanized melanocytes but in greater numbers than in naïve skin. Consistent with our previous observations, the resulting pigmentation after retreatment with UVR often showed darker perifollicular areas, in contrast to the even pattern produced by retreatment with a compound alone or a compound plus UVR.

Although our results suggest that the melanocyte is the main target for these compounds, no known melanocytic receptor for PIP or its derivatives has been identified to date. Interestingly, the presence of one of the subtypes of vanilloid receptor, the receptor for PIP and PIP-related molecules, has recently been shown in keratinocytes.[30] In this respect, it is well known that melanocytes and keratinocytes exhibit a close functional relationship. Keratinocytes are known to produce several factors that regulate melanocyte activity and survival, such as nerve growth factor, granulocyte-monocyte colony stimulating factor, basic fibroblast growth factor, endothelin-1, stem cell factor and other cytokines.[31–37] It has recently been shown that some of these molecules are imbalanced in vitiligo skin,[38] suggesting that the deregulation of the melanocyte microenvironment could be involved in the selective destruction of melanocytes in vitiligo. Indeed, an impairment of keratinocyte function is observed in perilesional skin.[39] It is reasonable to speculate that PIP and PIP analogues could have an effect on modulating cytokine production by keratinocytes *in vivo*, consequently stimulating melanocyte replication or activity, which could result in an increase in pigmentation. Nevertheless, we have observed an effect on PKC activation by PIP *in vitro* that is suggestive of a direct effect on melanocytes.[11]

In summary, we have shown that topical treatment with PIP, and two of its synthetic analogues, THP and rCHP, stimulates even pigmentation in mice. Topical treatment in combination with low dose UVR significantly enhances the pigmentation response with results that are cosmetically better compared with conventional vitiligo therapies when applied to mice. Although fading may occur when the treatment is interrupted, a good pigmentation response is readily achieved again after short periods of retreatment. Side effects, such as irritation and inflammation, were transient and tolerable. These data provide strong support for the future clinical evaluation of PIP and its derivatives as novel treatments for vitiligo.

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