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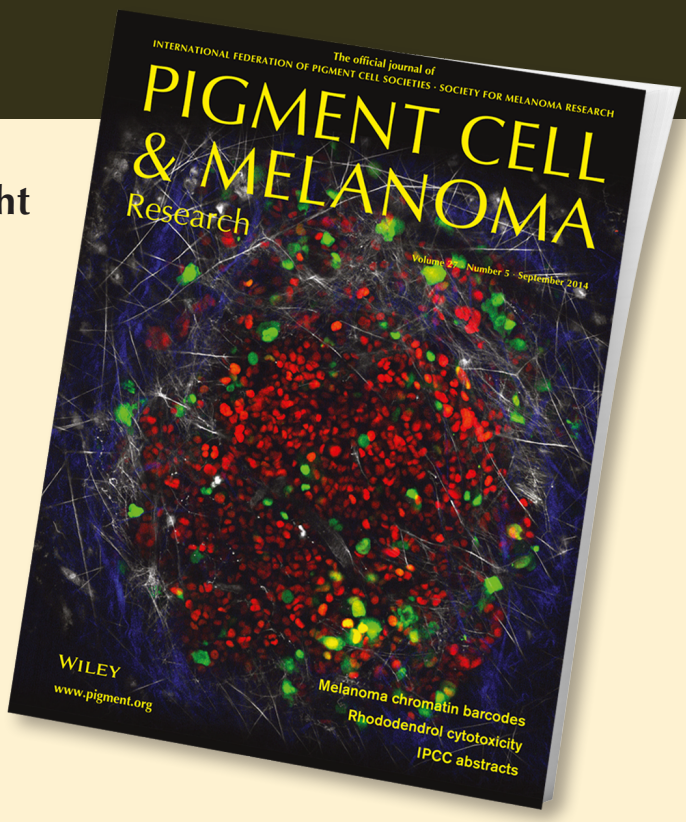

Pheomelanin-induced oxidative stress: bright and dark chemistry bridging red hair phenotype and melanoma

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DOI: 10.1111/pcmr.12262
Volume 27, Issue 5, Pages 721–733

Pheomelanin-induced oxidative stress: bright and dark chemistry bridging red hair phenotype and melanoma

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KEYWORDS red hair/pheomelanin/melanoma/oxidative stress/UV/glutathione/reactive oxygen species

PUBLICATION DATA Received 23 January 2014, revised and accepted for publication 8 May 2014, published online 11 May 2014

doi: 10.1111/pcmr.12262

Summary

The complex interplay of genetic and epigenetic factors linking sun exposure to melanoma in the red hair phenotype hinges on the peculiar physical and chemical properties of pheomelanins and the underlying biosynthetic pathway, which is switched on by the effects of inactivating polymorphisms in the melanocortin 1 receptor gene. In addition to the long recognized UV-dependent pathways of toxicity and cell damage, a UV-independent pro-oxidant state induced by pheomelanin within the genetically determined background of the red hair phenotype has recently been disclosed. This review provides a detailed discussion of the possible UV-dependent and UV-independent chemical mechanisms underlying pheomelanin-mediated oxidative stress, with special reference to the oxygen-dependent depletion of glutathione and other cell antioxidants. The new concept of pheomelanin as a 'living' polymer and biocatalyst that may grow by exposure to monomer building blocks and may trigger autooxidative processes is also discussed. As a corollary, treatment of inflammatory skin diseases in RHP patients is briefly commented. Finally, possible concerted strategies for melanoma prevention in the red hair phenotype are proposed.

Introduction: the red hair phenotype, UV susceptibility, and melanoma risk

Compelling epidemiological and clinical evidence indicates that the highest risk of developing melanoma, the deadliest highly metastatic form of skin cancer, is associated with the red hair phenotype (RHP) of Celtic origin (see e.g. Olsen et al., 2010) with pale skin, carroty or straw red hair, freckles, multiple nevi, blue eyes, and a high tendency to sunburn with an inability to tan. The RHP is characterized by the formation of the sulfur-containing reddish brown variant of melanin pigments, the pheomelanins (Prota, 1995; Thomson, 1974). The presence of these pigments in human hair but also in the epidermis has long been demonstrated (Thody et al., 1991). Several lines of evidence indicate that pheomelanins not only have a weak protecting capacity against UV radiation relative to eumelanins but, conversely, are highly phototoxic and can amplify UV-induced production of reactive oxygen species (ROS) (Chedekel et al., 1978; Panzella et al., 2010; Rouzaud et al., 2005; Takeuchi et al., 2004; Wenczl et al., 1998). Although UV-dependent pathways for the induction of melanoma have been identified (Noonan et al., 2012), corroborating a direct correlation between sun exposure and melanoma risk, UV-independent processes have also been implicated, as suggested by the incidence of the tumor in non-exposed areas of the body, at variance with non-melanoma skin cancers.

The discovery in 1995 that specific mutations at the melanocortin 1 receptor (MC1R) gene are responsible for the switch from eumelanin to pheomelanin underlying the RHP (Valverde et al., 1995) drew attention to the manifold consequences of altered MC1R signaling. *MC1R* encodes a cyclic AMP-stimulating G-protein-coupled receptor that controls pigment production. Impaired receptor activity, as in the red hair/fair skin mutants, produces the red/yellow pheomelanin pigment, whereas high levels of MC1R activity stimulate the production of black/brown eumelanin.

Genetic data demonstrated that certain allelic variants of MC1R associated with the RHP, namely R151C, R160W, and D294H, are related to increased melanoma susceptibility (Abdel-Malek et al., 2008). Nine most common MC1R variants have been shown to make a sizeable

contribution to the burden of melanoma, with the red hair color (RHC) variants having the highest risk (Williams et al., 2011). However, epidemiological studies showed that MC1R RHC and SCL45A2 p.P374L variants are strong melanoma risk predictors, notably in those individuals who would not be identified as high risk based on their phenotypes alone (Ibarrola-Villava et al., 2012). In addition, certain MC1R variants could increase melanoma risk due to their impact on pathways other than pigmentation and may be linked to specific melanoma subtypes (Puig-Butillé et al., 2013). For example, while p.R163Q is associated with lentigo maligna melanoma under a dominant model of heritance, no association was found between p.R163Q and Fitzpatrick skin phototype, eye color, or skin color. Recent evidence also showed that MC1R RHC variants in the presence of $BRAF^{V600E}$ fail to suppress PI3K/AKT signaling driving oncogenic transformation and melanomagenesis under UVB exposure (Cao et al., 2013).

MC1R exerts also controlling effects on ROS-dependent damage by increasing the levels of base excision repair enzymes 8-oxoguanine DNA glycosylase (OGG1) as well as DNA damage repair enzyme apurinic apyrimidinic endonuclease 1 (APE-1/Ref-1) (Kadekaro et al., 2012). Accordingly, a number of questions can be posed concerning the actual relationship between RHP and melanoma: is the RHP a simple marker of melanoma risk, or does the pigment pathway play a major role or contribute to some extent to the disease onset and progression? Is melanoma risk related to the intrinsic properties of phototoxic and carcinogenic pheomelanins or is it rather related to the lack of the photoprotective eumelanin pathway, chiefly of the diffusible antioxidant melanogen 5,6-dihydroxyindole-2-carboxylic acid (DHICA) (Kovacs et al., 2012; Panzella et al., 2011a)? Is the pheomelanin pathway a major culprit for melanoma development in RHP or is it only an innocent bystander, a mere epiphenomenon of the inactivation of MC1R-dependent pathways?

An important contribution toward settling these issues has come from the demonstration that the pheomelanin pigment pathway is implicated in *UV-radiation-independent* carcinogenic contributions to melanomagenesis by the mechanisms of oxidative DNA damage (Mitra et al., 2012). By using a conditional, melanocyte-targeted allele of the most common melanoma oncoprotein, $BRAF^{V600E}$, into red haired mice carrying an inactivating mutation in the Mc1r gene, a high incidence of invasive melanomas was observed without providing additional gene aberrations or ultraviolet radiation exposure. On the other hand, introduction of an albino allele, which deletes pigment production on the $Mc1r^{e/e}$ background, proved to be protective against melanoma development. In addition, normal $Mc1r^{e/e}$ mouse skin was found to have significantly greater oxidative DNA and lipid damage than albino- $Mc1r^{e/e}$ mouse skin.

Although caution should be exercised whenever extending data from animal models to man, the demonstration that melanoma risk in a mouse model of RHP is directly associated with the synthesis of pheomelanins and not with the lack of photoprotective eumelanins would draw renewed attention to the effects of the pigment pathway (Figure 1).

In a subsequent study (Morgan et al., 2013), two possible not mutually exclusive mechanisms by which the pheomelanin pathway could mediate oxidative stress and melanomagenesis were postulated (i) although pheomelanin is not located in the nucleus, it might cause damage by promoting the formation of ROS which could overwhelm cellular antioxidant reserves and cause oxidative damage to biomolecules including free nucleobases in the cytosol, (ii) the pheomelanin biosynthetic pathway depletes cysteine-based cellular antioxidants making the cell more vulnerable to elevated ROS levels. A number of issues remain to be defined: What are the mechanisms of UV-independent oxidative damage induced by pheomelanin? Does pheomelanin mediate ROS generation? If so, what are the structural elements and mechanisms responsible for ROS production? In this study, we overview the latest advances in the field of pheomelanin chemistry and photophysics with a view to providing a chemical framework into which to explain the possible mechanisms underlying the relationships between pheomelanin synthesis and oxidative stress. Putting together newly emerging concepts in the field of pigment synthesis and control, we also propose new possible guidelines for the development of targeted strategies for melanoma prevention.

Highlight 1. Induction of UV-independent melanoma is much higher in red mice compared with albino or black mice; levels of markers of ROS-mediated DNA damage are higher in red mice compared with albino mice, indicating that it is the pheomelanin pathway, rather

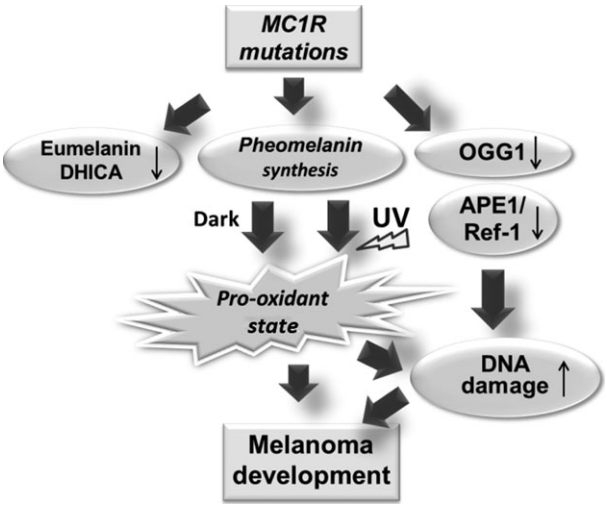

Figure 1. Some relationships between the Red Hair Phenotype and melanomagenesis.

than the lack of the eumelanin pathway, that is critical for oxidative stress, DNA damage and melanoma development.

Pheomelanin structure and properties

Pheomelanin formation is biochemically sustained by elevated levels of cysteine (Ito and Wakamatsu, 2011; Prota, 1995; Prota and Nicolaus, 1967) leading to 5-*S*-cysteinyldopa (5SCD) and minor amounts of 2-*S*-cysteinyldopa (2SCD) which on oxidation lead to benzothiazine intermediates undergoing dimerization, cross-coupling or ring contraction to benzothiazole units by complex, as yet poorly elucidated mechanisms (Greco et al., 2011b; Napolitano et al., 2001; Thureau et al., 2012; Wakamatsu et al., 2009). Most of these processes are favoured in the presence of zinc ions directing the oxidation of 5SCD toward the formation of the 3-carboxylated benzothiazine intermediate (Napolitano et al., 2001). Altogether, these molecular components may contribute to the overall chromophoric features and photoactivity of pheomelanins in the UV-visible region (Napolitano et al., 2008; Panzella et al., 2010; Ye et al., 2008).

Chemical degradation of pheomelanins leads to a series of fragments including 4-amino-3-hydroxyphenylalanine (AHP), a benzothiazole carboxylic acid derivative (BTCA), and a thiazolyl-pyridine pentacarboxylic acid (TPCA) (Figure 2). AHP is formed by reductive hydrolysis of benzothiazine but not benzothiazole units (Wakamatsu et al., 2009), BTCA results from the alkaline H_2O_2 degradation of benzothiazole or benzothiazine units (Greco et al., 2009; Panzella et al., 2007), and TPCA has recently been shown to derive from permanganate degradation of a dimeric benzothiazolyl-thiazinoisoquinoline intermediate (BT-TIQ) in the oxidative polymerization of 5SCD (Greco et al., 2011a) in the presence of zinc ions.

Interestingly, red hair pheomelanin gives relatively high yields of TPCA showing that the BT-TIQ units are dominant structural building blocks (Greco et al., 2012).

In addition to 5SCD, the isomer 2SCD is also an important building block of red human hair pheomelanin. Degradative studies have shown that the pigment suffers extensive modification during hair growth revealing different stability properties of its main components, whereas the major 5SCD-derived bulk component undergoes degradation, the 2SCD-derived core is relatively stable (Greco et al., 2009). Structural degradation occurs during hair growth probably as a result of oxidative processes related in part to sun exposure.

Highlight 2. Red human hair pheomelanin consists mainly of low molecular weight oligomers of benzothiazolylthiazinodihydroisoquinoline units, as well as of benzothiazine and benzothiazole units. An important contribution to the red hair pigment is due, besides 5-S-cysteinyldopa, to the 2-S-cysteinyldopa isomer.

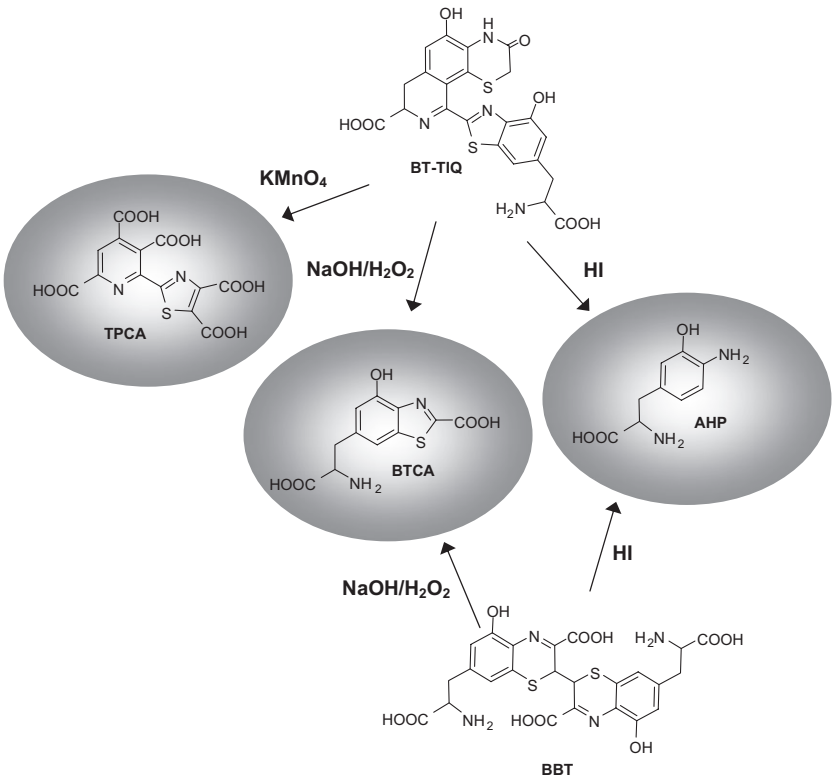

Figure 2. Main benzothiazine-related oligomers reflecting pheomelanin structural units and origin of the fragments derived by chemical degradation.

The bright chemistry of pheomelanin: phototoxicity and photodegradation

Traditionally, the positive correlation between RHP and melanoma has been attributed to the capacity of pheomelanin to act as a potent photosensitizer leading to intense production of ROS by UV-visible light irradiation (Bennett, 2008; Sarna et al., 1985; Ye and Simon, 2002). As the seminal works of Chedekel (Chedekel et al., 1978, 1980), it is commonly accepted that pheomelanin chromophore is excited to the singlet state, decays to the triplet, and transfers an electron to oxygen affording the superoxide anion (Figure 3), which can indirectly induce oxidative base lesions and DNA strand breaks (Wenczl et al., 1998) through its downstream products, such as hydrogen peroxide and hydroxyl radicals. Some evidence also indicates transfer of excitation from the pheomelanin to oxygen, giving singlet oxygen ($^1O_2$) which, besides being cytotoxic per se, is photochemically active leading to destruction of the pheomelanin chromophore (Korytowski et al., 1986). After UVA irradiation pheomelanin absorbance decreases both in the visible and the UVA range, consistent with the poor ability of photoprotection observed for people with light skin and red hair (Ou-Yang et al., 2004).

The mutagenic effects of UVB radiation and its direct implication in melanomagenesis has been amply documented while the role of melanin remains controversial, though a protective action is generally accepted in the case of eumelanin (Swalwell et al., 2012).

The picture is indeed much complex as epidemiological studies would indicate a statistically significant association between cumulative UV flux and cancer risk of non-melanoma skin cancers (NMSC), but less significant for melanoma (Wu et al.,2014). In squamous and basal cell carcinomas, UV-signature mutations, such as in the tumor suppressor p53, are more common than in melanoma. On the other hand, there is increasing evidence supporting the importance of oxidative DNA damage in melanoma formation, and the concept that melanoma is a tumor driven by reactive oxygen species. Dysplastic nevi that are precursors of melanoma have higher pheomelanin content and express more reactive oxygen radicals than normal human melanocytes (Pavel et al., 2004). Moreover, it has been proposed that the common somatic $BRAF^{V600E}$ is caused by increased generation of reactive oxygen radicals (Bauer et al., 2011). This mutation is significantly more prevalent in melanomas arising on non-chronically sun-exposed sites than on sun-exposed sites and is markedly increased in individuals with any MC1R variant. Overall, these data would point to a major implication of pheomelanin in melanoma not necessarily associated with UV exposure.

Histological and electron microscopic examination established that in dark skin melanosomes that are enriched in eumelanin remain intact and persist in the superbasal layers of the epidermis, while in lightly pigmented skin, melanosomes are degraded, leaving only 'melanin dust' in the upper epidermal layers and compromising the photoprotection of the skin (Brenner and Hearing, 2008). The presence of pheomelanin together with eumelanin in human epidermis has been demonstrated by Thody and coworkers (Thody et al., 1991). Since then most of the conclusion of studies on human hair have been extended to epidermal melanins that occur in the tissues at exceedingly low levels preventing direct structural investigation. The difficulties in determining the actual presence of pheomelanin in RHP skin is clearly indicated by the controversial picture that emerged even in the analyses of cultured melanocytes from donors of different phenotypes with MC1R loss-of-function mutations (Wakamatsu et al., 2006).

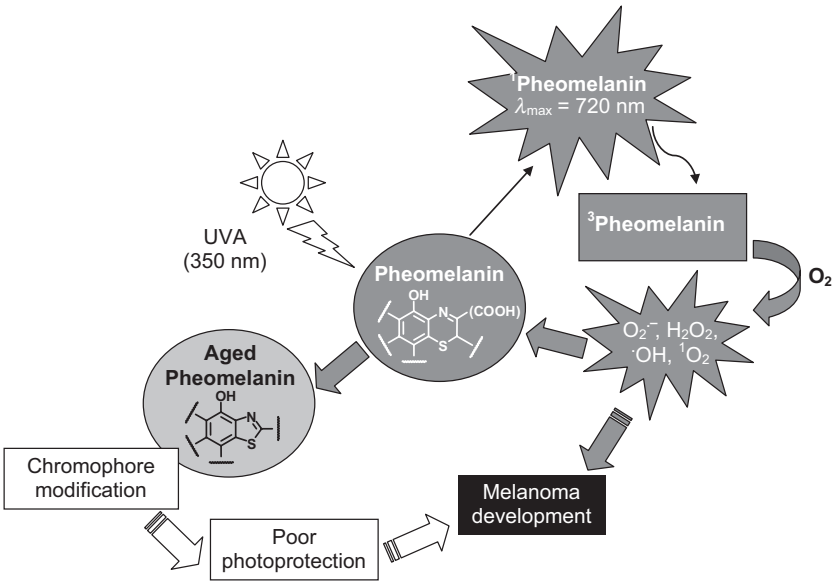

Figure 3. UVA photoexcitation of benzothiazine chromophores within pheomelanin results in ROS generation from oxygen and modification of the pigment structural units with decrease in the photoprotective action. Excited-state pheomelanin (singlet pheomelanin, ^{1}Pheomelanin) exhibiting an absorption maximum in the low energy red range of visible light decays to ground state (triplet pheomelanin, ^{3}Pheomelanin), a species capable of reducing molecular oxygen with production of ROS.

Prolonged exposure of red hair locks to sunlight caused a substantial structural degradation of 5SCD-related melanin units as a result of oxidative processes promoting conversion of the benzothiazine moieties to benzothiazoles (Greco et al., 2009; Wakamatsu et al., 2012). This process is expected to modify the pigment chromophore lowering the ability of photoprotection (Figure 3). Thus, taking hair melanin as a valuable model of epidermal pigments, light-induced structural modifications would result in significant changes of the chromophoric properties and the associated photosensitizing ability of these pigments. In line with this view, extensive structural modification of pheomelanin due to the lifelong exposure to blue light was also recently reported in human retinal pigment epithelium (Ito et al., 2013).

Apart from generating ROS, UVA irradiated synthetic pheomelanin was found to induce charge modifications on native catalase in reconstructed epidermis by a mechanism involving singlet oxygen (Maresca et al., 2006). In addition, an atypical mode of cell death in murine skin was discovered, in which UV-irradiated pheomelanin photosensitizes adjacent cells to apoptosis (Takeuchi et al., 2004). Care should be taken, however, in extrapolating the results on animal models of photocarcinogenesis to the human case, as animals vary in the distribution of melanocytes between the epidermis, dermis and hair follicles, the epidermal thickness (affecting UV penetration), the efficiency of DNA repair, and so on (Bennett, 2008). Moreover, recessive *Mc1r* yellow mice synthesize mostly pheomelanin (Hirobe et al., 2013; Lamoreux et al., 2001; Wolnicka-Glubisz et al., 2013), while human melanocytes synthesize both pheomelanin and eumelanin (Wakamatsu et al., 2006), the latter of which might counteract the effects of pheomelanin by scavenging ROS (Abdel-Malek and Ito, 2013). Actually, no significant relationship was found between minimum erythemal dose (MED) values and pheomelanin, eumelanin or total melanin content; a positive correlation was found, however, between the eumelanin/pheomelanin ratio and the MED values, suggesting that a high UV sensitivity is associated with high pheomelanin and low eumelanin levels (Vincensi et al., 1998). In this line, some recent studies (Hauser et al., 2006; Robinson et al., 2010) indicated that lower levels of eumelanin in RHP individuals may be more important than higher levels of pheomelanin in inducing DNA damage and thence melanomagenesis, and that MC1R may play a role in UV protection not related to pigmentation. Spontaneous tumor formation was observed in pigmented, but not in albino HGF mice by Noonan et al. (2012). Melanoma tumor formation was ascribed to endogenous oxidation resulting from dysregulated melanin synthesis in the absence of UV exposure, a status exhacerbated by UVA that is known to increase generation of reactive oxygen species.

Consistent with a greater photoreactivity, pheomelanosomes from red human hair exhibited a lower ionization potential with respect to eumelanosomes: the photoionization threshold of pheomelanin is ca. 326 nm, that is in the UVA region of the spectrum, whereas photoionization threshold of eumelanin falls in the UVB region (ca. 280 nm) (Ye et al., 2006). Consistently, a clear increase in oxygen uptake by pheomelanin irradiated between 338 and 323 nm was observed in EPR oxymetry measurements.

With regard to the structural factors determining pheomelanin photoreactivity, ultrafast absorption spectroscopy of synthetic pheomelanin at pH 7.2 revealed a transient species (720 nm) following UVA excitation at 350 nm, which was assigned to the excited-state absorption of the first excited singlet state of pheomelanin (Figure 3) (Ye and Simon, 2002). Pump-probe spectroscopic measurements suggested the involvement of benzothiazine units with absorption spectra peaking in the range from 350 to 360 nm, such as the 2,2′-bibenzothiazine dimers (BBT, Figure 2) from 5SCD, as the reactive chromophore in pheomelanin photophysics (Ye and Simon, 2003; Ye et al., 2008). Also trichochromes, the low molecular weight components of pheomelanin with absorption in the visible region, were shown to access their multiple electronic states and particularly a long-lived excited state upon visible light excitation (Ye et al., 2003), but unlike polymeric pheomelanins, they exhibited little aerobic photoreactivity and photogenerated superoxide anion at a very low yield (Simon et al., 2006).

In a recent study, the effects of zinc ions in enhancing both oxygen consumption and superoxide production in model pheomelanin pigments following irradiation with UVA and visible light was described (Panzella et al., 2010), pointing to BT-TIQ, BBT, and all the structural units derived from carboxylated benzothiazines (Greco et al., 2011a) as the main structural determinants of pheomelanin phototoxicity. Of course, caution is urged in deriving insights from synthetic pigments to account for the behavior of natural systems, as melanin structure and properties, for example, metal binding, ROS generation and scavenging, may result from how the pigments are assembled in the melanosomes. In addition, whether redox active metal ions commonly present in natural melanins (Liu et al., 2005) played any role in the phototoxicity of pheomelanin is difficult to assess. For example, Krol and Liebler (1998) reported that the prooxidant activities of pheomelanin on UV-induced liposomal lipid peroxidation result from pheomelanin-metal complexation. This should be an important focus of future studies.

> Highlight 3. UV-visible light irradiation of pheomelanin leads to reactive oxygen species that may damage the pigment itself. These effects are enhanced in the pigments prepared in the presence of zinc ions indicating benzothiazolylthiazinodihydroisoquinolines and all the structural units derived from 3-carboxy benzothiazines that show absorption in the UVA and visible region as the main structural determinants of the pigment photoreactivity.

The dark chemistry of pheomelanin: prooxidant and redox properties

Despite the demonstration of UV-independent mechanisms of melanoma development in a pheomelanin background in mice (Mitra et al., 2012), the detailed mechanisms by which pheomelanin can induce a prooxidant state in the absence of UV stimulation are little understood. The effects of the pigment could be explained by two major interrelated, mutually non-exclusive mechanisms, which may act in concert, that is, (i) enhanced ROS formation or (ii) depletion of major antioxidants.

That pheomelanin can produce ROS under UV-independent conditions is an intriguing point. If pheomelanin is equilibrated with air, no spontaneous electron transfer to oxygen to generate ROS would occur, because the generation of superoxide and other ROS requires a reductive input, that is, electron transfer from pheomelanin. It has been suggested that the lower ionization potential of pheomelanin (i.e., the energy required to remove an electron from the molecule), favoring formation of sulfur-stabilized radical cations, and the lower melanosomal pH of pheomelanin synthesis than eumelanin synthesis play some role in promoting ROS formation (Mitra et al., 2012). This suggestion, though chemically questionable due to the lack of specific evidence for sulfur-stabilized radical cations in pheomelanins, would be consistent with the remarkable ability of the benzothiazines constituents of pheomelanins to undergo H-atom abstraction and free-radical dimerization upon protonation (Leone et al., 2013; Napolitano et al., 2013) (Figure 4).

The ability of pheomelanin to directly induce antioxidant depletion has been disclosed in a recent paper (Panzella et al., 2014). In this study, it is shown that the purified pigment from red human hair (d'Ischia et al., 2013) markedly accelerates the autooxidation of two important cellular antioxidants and reducing agents, that is, GSH and NAD(P)H, with respect to purified black human hair eumelanin. A similar accelerating effect was caused by synthetic pheomelanin from 5SCD. This effect depends on oxygen and is not affected by superoxide dismutase (SOD) or catalase as ROS scavengers. As no detectable levels of ROS were observed under the reaction conditions with a variety of methods, including EPR and spin trapping or colorimetric assays (Chedekel et al., 1978; Panzella et al., 2010), it was argued that the accelerating effect of pheomelanin is due to the property of the pigment to serve as a redox catalyst accepting H-atoms from the substrates and transferring electrons to oxygen. Operation of a direct H-atom transfer to pheomelanin was confirmed by EPR analysis showing a selective decrease in the pheomelanin signal in purified red hair pigment following incubation with excess GSH (Panzella et al., 2014).

The ability of pheomelanin to markedly accelerate autooxidation reactions compared with eumelanins is

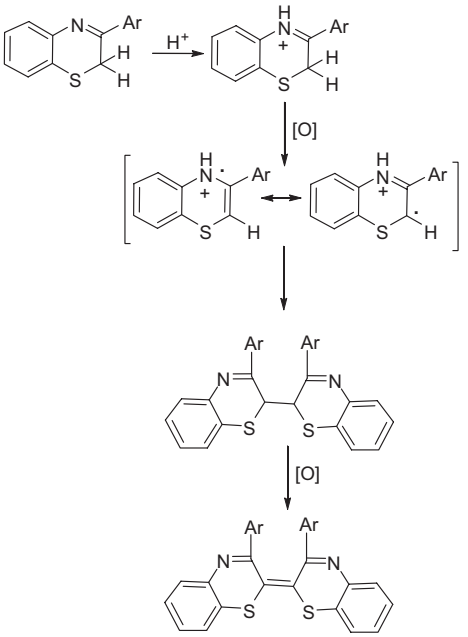

Figure 4. H-atom abstraction from the 1,4-benzothiazine system and oxidative radical coupling to the $\triangle^{2,2'}$-bibenzothiazine is driven by protonation.

apparently in contrast with the oxidation potentials measured for human eumelanosomes and pheomelanosomes (Samokhvalov et al., 2005), which suggested conversely a stronger reducing capacity of the latter. It is possible that both oxidized and reduced moieties coexist in pheomelanin and that the reported oxidation potentials reflect an average property of the pigment while chemical reactions depend on reactivity at specific oxidized units, but this requires verification. As mentioned previously, interaction of GSH with pheomelanin seems to proceed with a direct H-atom transfer from the thiol to free radical moieties of the pigment. Reduced pheomelanin thus formed would be efficiently reoxidized by oxygen, generating ROS and restoring the free-radical population. It has been suggested that the free-radical signal of red hair pheomelanin reflects an *o*-aminophenol/*o*-quinoneimine = 2 semiquinoneimine equilibrium within the pigment (Chio et al., 1982; Sealy et al., 1982) (Figure 5) akin to the catechol/*o*-quinone= 2 semiquinone comproportionation equilibrium of eumelanins (Mostert et al., 2012; Pezzella et al., 2013) and that GSH shifts the equilibrium toward the *o*-aminophenol component by H-atom transfer to the semiquinoneimine. In this mechanistic scheme, ROS would be produced from re-oxidation of pheomelanin by oxygen but would be immediately utilized in the redox cycle.

Notably, the pheomelanin-like polymer from the model compound 5-S-cysteinylmethylcatechol (unpublished), which lacks isoquinoline moieties (Greco et al., 2011a), exerts an accelerating effect similar to that of red hair pheomelanin, suggesting that the prooxidant properties

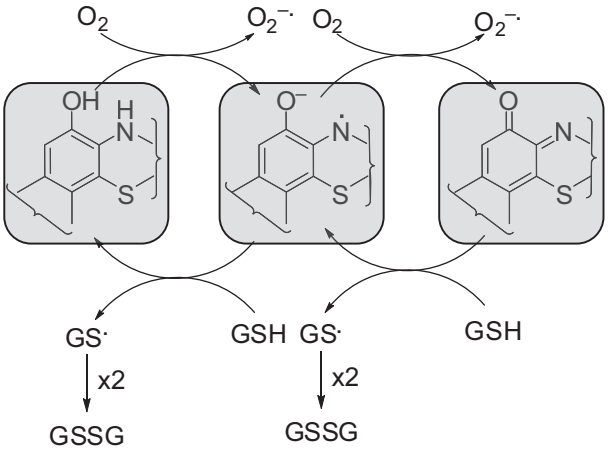

Figure 5. Redox equilibria between pheomelanin reactive moieties.

of natural and synthetic pheomelanins are independent of the isoquinoline moiety.

Besides inducing antioxidant depletion, red hair pheomelanin, like synthetic 5SCD melanin, proved also capable of promoting autooxidation of melanin precursors, such as 5SCD and dopa, under conditions of rigorous exclusion of light and at physiological pH, with the formation of eumelanin or pheomelanin pigments coating preformed pheomelanin particles (Greco et al., 2011b; Panzella et al., 2014). This effect is oxidative in character, because it depends on oxygen, revealing a remarkable prooxidant capacity of the pigment serving as a biocatalyst for non-enzymatic melanogenesis (Figure 6). The prooxidant effect can likewise be ascribed to a direct interaction of the catechol substrate with pheomelanin, as evidenced by the lack of effect of SOD or catalase. The property to enhance UV-independent autooxidation reactions is specific of pheomelanin, because black human hair eumelanin is completely ineffective and has no parallel among natural pigments and biopolymers. It is probably linked not only to the intrinsic redox features of pheomelanin structure, but also to its partial solubility in neutral aqueous media and its ability to chelate and retain transition metal ions, also as impurities in the medium (Liu et al., 2005). Eumelanin, conversely, gives rise to small, compact, and structured aggregates from extended, highly conjugated and tightly stacked molecular components (Panzella et al., 2013) compared with pheomelanin which would make the pigment less available for redox interactions.

The ability of pheomelanin to induce the rapid depletion of monomer precursors in solution via an oxygen-dependent process opens new scenarios in the mechanisms of melanogenesis. This phenomenon might entail hitherto unrecognized surface properties of pheomelanins denoting a 'living polymer'-like behavior, whereby rapid pigment growth may occur by simple exposure to monomer precursors in the presence of oxygen. Based on available evidence, it can be speculated that melanin synthesis in pheomelanin-forming melanocytes may be the result at least in part of a non-enzymatic process promoted by even small amounts of pigment. As soon as produced, pheomelanin may initiate oxygen-sustained redox exchange with enzymatically produced dopa or 5SCD causing deposition of eumelanin or pheomelanin depending on the relative availability of the precursors. This mechanism not only explains in a convincing manner the origin of the casing pheomelanin core/eumelanin shell architecture in mixed melanins (Ito, 2006; Peles et al., 2009) but settles also the fundamental issue of what oxidizing system brings about pheomelanin synthesis, as it is known that the committed enzyme of melanogenesis, tyrosinase, is unable to oxidize 5SCD and benzothiazine intermediates thereof (Hansson et al., 1980).

> Highlight 4. Red human hair pheomelanin and synthetic pheomelanin markedly accelerate GSH and NAD(P)H autooxidation in the presence of oxygen by a direct H-atom transfer mechanism not mediated by ROS. By similar mechanisms, pheomelanin promotes autooxidation of dopa and 5-S-cysteinyldopa to form melanin coatings on suspended pigment. This latter process explains both the origin of the casing architecture of mixed melanins and the mechanisms of pheomelanogenesis in the absence of enzymatic assistance.

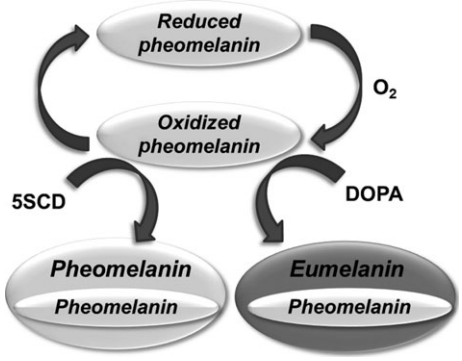

Figure 6. Pheomelanin triggered melanogenesis.

A unifying view of pheomelanin-dependent mechanisms of oxidative stress in RHP

A number of critical issues about pheomelanin-induced oxidative stress, some of which have been posed in a previous paper (Morgan et al., 2013), are addressed in the following.

1. What is the relative contribution of light-induced and dark pheomelanin-mediated oxidative processes?

While both processes are likely to contribute to pheomelanin-induced carcinogenesis, they appear to play

different roles on different temporal scales. UV-induced photochemical processes can induce more profound oxidative damage and should be responsible for acute oxidative stress, sunburn, and skin cancer, as they cause a burst of ROS overwhelming the primary antioxidant defence of the skin. On the other hand, UV-independent prooxidant reactions are less efficient than sun-induced ones and account for a permanent abatement of the baseline antioxidant defense favouring the onset of pro-inflammatory conditions and exacerbating the outcome of severe phototoxic events.

2. What is the energy input for direct ROS production by pheomelanin and what are the structural units involved?

Production of ROS requires transfer of energy or of electrons to molecular oxygen. Energy transfer requires that a photosensitizing chromophore in its excited state undergoes efficient intersystem crossing to generate a triplet state that can exchange energy with ground state triplet dioxygen to produce singlet oxygen, a highly reactive electronically excited form of oxygen. At present, only circumstantial evidence exists for singlet oxygen production by photoexcitation of pheomelanin with UVA (Maresca et al., 2006). Conversely, there is ample evidence that the most significant consequence of pheomelanin irradiation is the production of superoxide and related oxygen radicals via electron transfer from excited states of the pigment (Panzella et al., 2010). Thus, electron transfer is a fundamental energy input for ROS production via photoexcitation. No evidence has so far been obtained for superoxide or other ROS formation from human hair pheomelanin via electron transfer from GSH or other reducing agents in the dark. However, as it has been demonstrated that GSH can reduce pheomelanin, formation of ROS by re-oxidation of reduced pheomelanin by oxygen is likely to be an important event ensuring shuttling of pheomelanin between the redox states. Mechanistically, it is conceivable that the two main inputs for ROS production by pheomelanin, that is, UV-dependent and UV-independent electron transfer/reduction, involve different reactive moieties of the pigment. The enhanced photoreactivity of pheomelanins prepared by 5SCD oxidation in the presence of zinc ions, under which conditions the 3-carboxybenzothiazine is primarily formed, would suggest that BT-TIQ and BBT structural units, arising from the oxidation of this benzothiazine intermediate and conferring the pigment the visible and UVA absorption features, are mainly involved in the light dependent ROS production. On the other hand, the oxidizing activity was found to be higher for the synthetic pheomelanins prepared in the absence of zinc ions compared with that obtained in the presence of zinc ions (unpublished) which would point to non-carboxylated benzothiazine intermediates as the active structural units in ROS production by pheomelanin in the dark. Accordingly, this activity is exhibited also by the pigment obtained from a model catechol that cannot give rise to BT-TIQ type units (Figure 7).

3. Can pheomelanin actually induce UV-independent GSH depletion in vivo?

Although pheomelanin is chemically competent to promote GSH oxidation in vitro, an issue is raised of how efficient such an interaction may be within the melanocyte where the pigment is highly compartmentalized within the melanosomes. It is known that pheomelanosomes are less regularly shaped and compact than eumelanosomes. They may appear as ellipsoids and spheres showing microvesicular and proteinaceous matrices on which melanin deposition is spotty and granular and are structurally degraded upon extraction from the keratin matrix (Jimbow et al., 1983; Liu et al., 2005). Once pheomelanosomes are mature, they are transferred to keratinocytes, where they are available to enter into contact with major cellular ingredients including GSH, NADH, and other redox-active biomolecules. Thus, the pigment component of pheomelanosomes is likely to be

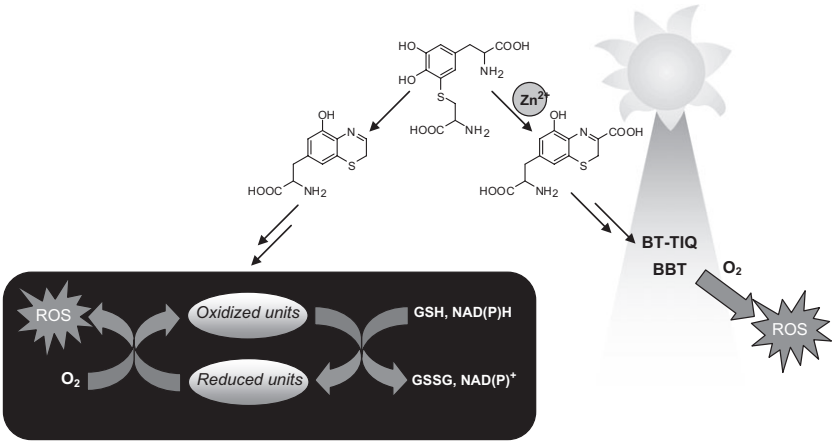

Figure 7. UV-dependent and UV-independent electron transfer/reduction mechanisms of ROS production by pheomelanin. Shown are the two benzothiazine monomers precursors to the active structural units within the pigment and the proposed key role of zinc ions.

more accessible than that of the eumelanosomes and may be available to induce a depletion of cellular antioxidants at micromolar concentrations. Furthermore, it could be speculated that the apparently damaged surface of pheomelanosomes is the results of ROS production following interaction of the pigment with GSH.

Another mechanism of antioxidant depletion that has been recently implicated to account for oxidative stress is the synthetic pathway of pheomelanogenesis per se, which consumes cysteine with consequent depletion of GSH (Galván et al., 2012a,b,c; Galván and Møller, 2013; Morgan et al., 2013). Utilization of cysteine is a normal regulatory mechanism of melanogenesis based on the scavenging of dopaquinone to produce diffusible cysteinyldopas. This process is well detectable in all skin types in the form of urinary excretion of cysteinyldopas. However, in adequately photoprotected dark phenotypes, under conditions of redox control ensured by the eumelanin pathway, including DHICA (Kovacs et al., 2012; Panzella et al., 2011a), most if not all of the 5SCD is excreted from the melanocyte without entering melanogenic pathways. On the other hand, in the prooxidant background of the poorly photoprotected RHP, 5SCD is engaged with oxidative reactions leading to pheomelanin accumulation which in turn amplifies antioxidant depletion and oxidative stress. Thus, the two main mechanisms that consume GSH stores are mutually reinforced in the RHP.

4. What are the actual UV-independent mechanisms underlying biological endpoints of the prooxidant state within the RHP, for example, enhanced lipid peroxidation and oxidative DNA damage (Mitra et al., 2012)?

In principle, both antioxidant depletion and ROS production by pheomelanin may be responsible for DNA damage and lipid peroxidation; however, the former seems to play a major role (Figure 8).

The decreased ability of pheomelanin-producing melanocytes to cope with oxidative stress induced by both endogenous and exogenous ROS may account for increased DNA damage. Enhanced lipid peroxidation may likewise be an indirect consequence of the depletion of GSH which is no longer available to restore tocopherol levels in membranes counteracting free-radical peroxidation process affecting polyunsaturated lipids. Also, the observed abatement of the melanin precursor pool by pheomelanin, besides the relevance to melanogenesis, may play a contributory role in determining the prooxidant background of red hair/pale skin. 5SCD is a potential antioxidant serving as H-atom donor and a metal-chelating agent (Napolitano et al., 1996) which is produced at higher levels following sun exposure, inflammation, and a number of disease states (Murakami et al., 2007; Panzella et al., 2011b). Circulating 5SCD levels are also abnormally high in melanoma patients (Wakamatsu et al., 2002). Spontaneous pheomelanin growth by the oxidation of 5SCD induced by preformed pheomelanin may be seen as a threatening mechanism by which a

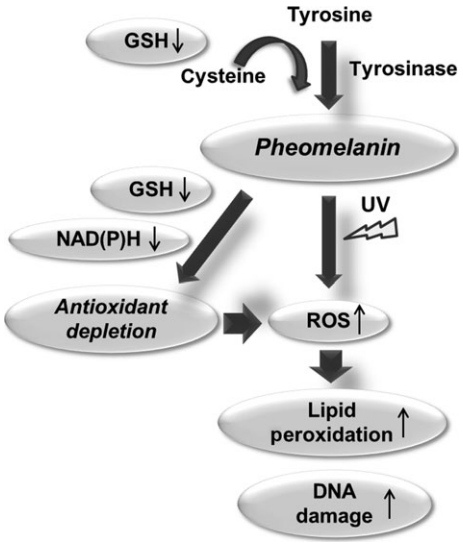

Figure 8. Overall view of the role of pheomelanin in triggering an oxidative stress under light exposure or dark conditions.

specific antioxidant defense of melanocytes is consumed to produce a toxic pigment. The burst in the ROS levels associated with sun exposure and pheomelanin photosensitization may dramatically accelerate the oxidative processes sustained by the pigment itself in the dark, thus amplifying DNA damage and lipid peroxidation as hallmarks of photoaging and carcinogenesis (Hadshiew et al., 2000; Terra et al., 2012).

It is worth noting that adverse oxidative stress-related conditions associated with the pheomelanic background have been described also in non-human mammals (Galván et al., 2012a,b,c) and in birds (Galván et al., 2012b), suggesting a broader relevance of pheomelanin-induced prooxidant state.

> Highlight 5. Oxidative stress, DNA damage, and lipid peroxidation in the red hair phenotype are likely to result primarily from antioxidant depletion derived both by direct interaction with the pigment and by the pigment pathway which consumes GSH stores to provide the cysteine for cysteinyldopa synthesis. Pheomelanin-induced autooxidative consumption of pigment precursors with antioxidant properties leading to new pheomelanin operating as pro-oxidant is an additional mechanism contributing to oxidative stress. Production of ROS by pheomelanin is likely to be a primary contributory factor to oxidative stress only under conditions of UV stimulation.

Inflammatory skin diseases: role of oxidative stress and possible difficulties in the treatment of RHP patients

Oxidative stress plays a pivotal role in the pathogenesis of different inflammatory skin diseases such as psoriasis,

acne, rosacea, contact dermatitis, and atopic dermatitis (Bickers and Athar, 2006; Briganti and Picardo, 2003).

Moderate amounts of ROS are crucial in cell regulation as they act as mediators and signal transduction molecules. Nevertheless, an excessive generation of ROS, driving the redox balance toward a pro-oxidant status, induces oxidative damage, and apoptotic pathway. Therefore, inflammatory skin diseases stem not only from an alteration of the cell oxidative status, but also from secondary responses induced by ROS and the interferences with the expression of the redox-sensitive transcription factors.

Several studies demonstrated that the activity of SOD, CAT, or GSH is lowered in patients suffering from severe forms of acne, psoriasis, and rosacea in comparison with healthy volunteers (Abdel Fattah et al., 2008; Gabr and Al-Ghadir, 2012; Kim et al., 2011; Oztas et al., 2003; Perihan et al., 2012; Sarici et al., 2010). ROS may participate in the pathogenesis of allergic reactions in the skin. Recently, it was shown that Th1 and Th2 response patterns in antigen presenting cells are modulated by GSH (Kaur et al., 2001). Atopic dermatitis (AD) is an inflammatory skin disease characterized by an overactivation of the Th2 response leading to the overproduction of IL-4, IL-5, and INF-γ. It has been suggested that an impaired homeostasis of oxygen/nitrogen radicals associated with an increased oxidative stress, measurable as urinary 8-hydroxy-2′-deoxyguanosine excretion, could be involved in the pathogenesis of AD. Interestingly *N*-acetyl-L-cysteine, a precursor of GSH, is able to downregulate the above-mentioned Th2 cytokines, suggesting that it could represent a new therapeutic approach to Th2-related disorders (Bengtsson et al., 2001).

Altogether these data point to the imbalance between the pro-oxidant and antioxidant stimuli as a critical factor in the pathogenesis of inflammatory skin diseases. Thus, it is expected that depletion of antioxidants in patients suffering from one of the above mentioned skin diseases is much more marked in RHP subjects because of the chronic oxidative stress associated with such pigmentation background. Therefore, treatment of these individuals, in particular of patients suffering from acne or AD, poses several problems and in most cases, in the authors' experience, the conventional therapeutic approaches prove inadequate to counteract the inflammatory processes involved in the development of the clinical picture of such diseases. Studies aimed at comparing patients with RHP and other phenotypes suffering from such inflammatory cutaneous conditions in terms of both clinical relevance and biochemical signatures would be highly desirable and could provide the basis for the development of *ad hoc* therapeutic strategies.

Highlight 6. The pathogenesis of many inflammatory skin diseases, such as psoriasis, acne, rosacea, contact dermatitis, and atopic dermatitis, involves oxidative stress conditions and an antioxidant imbalance. Treatment of such diseases in RHP patients that show chronic oxidative stress conditions may prove most difficult and may require different therapeutic strategies.

From pigment pathways to chemoprevention strategies

An excellent critical review on the possible relationships between RHP, oxidative stress, *MC1R* mutations, and melanomagenesis has been provided recently (Abdel-Malek and Ito, 2013) and it is out of the scope of this paper to address in detail such relationships. Although aberrant pigment pathways are not most likely the sole culprit in melanoma development, they provide a potentially useful target for controlling oxidative stress and tumor development. The rationale that may inspire potential chemopreventive strategies is built on a series of relevant observations (i) pheomelanin has more detrimental effects than the total absence of melanin, as in the albino mice (Mitra et al., 2012), (ii) the RHP lacks efficient α-MSH signaling, which entails a decreased ability of melanocytes to inhibit ROS generation and to enhance activity and expression of antioxidant enzymes, (iii) melanoma formation is associated with dysregulated melanin synthesis in the absence of UV exposure, and (iv) dysregulation is exacerbated by UVA increasing the generation of ROS (Noonan et al., 2012). Overall, these observations call into play the two main factors underlying melanomagenesis, that is, an aberrant pigment pathway and oxidative stress. These can be targeted simultaneously by a rational approach aimed at restoring 'functional' melanogenesis, that is, eumelanogenesis, by acting downstream of the dysfunctioning signaling pathway, and at recovering the antioxidant defense pool of melanocyte. A viable strategy toward this dual goal may be based on the joint administration of eumelanin precursors with potent antioxidant properties and a pharmacologically effective GSH precursor aimed at addressing mitochondrial dysfunction, a typical correlate of oxidative stress-based disease states. DHICA is a major precursor of natural eumelanins and has been shown to confer potent antioxidant capacity to the pigment (Jiang et al., 2010; Panzella et al., 2013). It has recently been recognized as a most potent free-radical scavenger and central mediator in cell/cell communication (Kovacs et al., 2012; Panzella et al., 2011a). Use of *N*-acetylcysteine as GSH precursor has been explored in melanoma with positive results in terms of control of UV-induced oxidative stress with delayed onset of melanocytic tumors in mice (Cotter et al., 2007; Meierjohann, 2014).

As pointed out recently (Abdel-Malek and Ito, 2013), caution should be exercised before extrapolating data from mice to humans. As melanin synthesis seems to be

aberrant in nevi and in melanoma tumors, we concur with previous authors (Abdel-Malek and Ito, 2013) that a detailed investigation of the melanin pathways in different skin types, in exposed versus non-exposed skin, and in normal skin versus melanoma tumors or nevi may be an important goal toward a definitive understanding of the role of the melanin pathway in melanomagenesis in highly susceptible individuals.

Highlight 7. The phenomena associated with melanomagenesis that is an aberrant pigment pathway and oxidative stress would suggest chemopreventive strategies aimed at restoring 'functional' melanogenesis, that is, eumelanogenesis, by acting downstream of the dysfunctioning signaling pathway, and at recovering the antioxidant defense pool of melanocyte.

Acknowledgements

This study was supported by a grant from Italian MIUR (PRIN 2010-2011- 010PFLRJR 'PROxi' project) and was carried out in the frame of the EuMelaNet program (http://www.espcr.org/eumelanet).

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