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Skin pigmentation enhancers

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Abstract

The highest incidences of cancer are found in the skin, but endogenous pigmentation is associated with markedly reduced risk. Agents that enhance skin pigmentation have the potential to reduce both photodamage and skin cancer incidence. The purpose of this review is to evaluate agents that have the potential to increase skin pigmentation. These include topically applied substances that simulate natural pigmentation: dihydroxyacetone and melanins; and substances that stimulate the natural pigmentation process: psoralens with UVA (PUVA), dimethylsulfoxide (DMSO), L-tyrosine, L-Dopa, lysosomotropic agents, diacylglycerols, thymidine dinucleotides, DNA fragments, melanocyte stimulating hormone (MSH) analogs, 3-isobutyl-1-methylxanthine (IBMX), nitric oxide donors, and bicyclic monoterpene (BMT) diols. These agents are compared with regards to efficacy when administered to melanoma cells, normal human epidermal melanocytes, animal skin, and human skin. In addition, mechanisms of action are reviewed since these may reveal issues related to both efficacy and safety. Both dihydroxyacetone and topically applied melanins are presently available to the consumer, and both of these have been shown to provide some photoprotection. Of the pigmentation stimulators, only PUVA and MSH analogs have been tested extensively on humans, but there are concerns about the safety and side effects of both. At least some of the remaining pigmentation stimulators under development have the potential to safely induce a photoprotective tan. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Solar radiation is a risk factor for skin cancer [1–3], but endogenous pigmentation provides protection against skin cancer and against DNA damage. Whereas the Caucasian population is prone to developing skin cancer as a result of sun exposure, the Black population is inherently resistant. Even though black skin provides an intrinsic SPF (sun protection factor) of only 3–5, much less than most sunscreens, the frequency of melanoma occurrence in African Americans is only 8% of that in Caucasians, and the frequency of nonmelanoma skin cancers is only 1% of that in Caucasians [4,5]. There may be several reasons for this, including: (i) unlike sunscreens, melanized skin provides broad wavelength protection from solar radiation; (ii) melanized skin exhibits greater immediate and delayed tanning reactions; (iii) melanin is thought to scavenge free radicals generated in skin by sun exposure; and (iv) protection in melanized skin does not rely upon application of a protective agent each time sun exposure is anticipated

[4,6]. If natural pigmentation could be safely induced before sun exposure, then many of the protective benefits of naturally melanized skin may be realized. Despite the fact that endogenous pigmentation has been associated with reduced cancer risk, it has been difficult to prove that induced pigmentation likewise reduces cancer risk. Part of the reason for this may stem from the fact that several key studies have induced melanogenesis via ultraviolet radiation (UVR) or UVR plus psoralen [7,8]. Both UVR and psoralen are mutagenic and associated with increased development of skin cancer [9]. Although it has not been demonstrated that induced pigmentation provides protection from development of cancer, it has been demonstrated that induced pigmentation provides protection against UVR-induced formation of DNA photoproducts [10–13]. Since DNA photoproducts lead to mutations and initiation of cancer, it is reasonable to suspect that stimulation of pigmentation by agents that do not damage DNA would provide protection from development of skin cancer. An artificial way to add protection for Caucasian skin is the use of sunscreens. Although sunscreens are generally believed to do much to prevent skin damage and skin cancer, there is recent evidence to indicate that they may

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not be as protective as originally thought. It is well established that sunscreens designed to prevent penetration of both UVA and UVB solar radiation are effective in preventing sunburn [4]. However, it is the conclusion of several epidemiology studies that sunscreens, by providing a false sense of security, can result in increased sun exposure [14–16]. Furthermore, sunscreens failed to protect mice from UV-induced melanoma development, even though these sunscreens did protect against sunburn cell formation and inflammation [17]. The hypothesis has been put forward that sunscreens are more effective blockers of the erythemic action spectrum than the melanoma action spectrum [16]. Since endogenous pigmentation provides broad wavelength protection from solar radiation, sunscreens used in combination with artificially induced tanning agents may provide more complete protection. In order to achieve this, one has to understand the biochemistry of natural tanning. The natural tanning process occurs in response to exposure to UVR. This process is characterized by increased number of melanocytes in the basal layer of the epidermis, increased size and number of melanosomes, increased production of melanin in melanosomes, increased dendricity of melanocytes, increased transport of melanosomes from melanocytes to keratinocytes, increased proliferation of keratinocytes, and thickening of the epidermis and stratum corneum [6,18]. Melanosomes are deposited over nuclei in keratinocytes, resulting in the formation of supranuclear caps that are characteristic of both sun-tanned skin and endogenous pigmentation [19,20]. These supranuclear caps provide protection from subsequent UVR exposure by scattering incoming light, and absorbing UVR-generated free radicals in cells. Photoprotection is also provided by thickening of the stratum corneum [11]. The rate-limiting enzyme in melanogenesis is tyrosinase [6,18]. This enzyme catalyzes the conversion of tyrosine to L-Dopa, and then dopaquinone. Subsequently, dopaquinone is converted either to brownish-black eumelanins, or reddish-yellow pheomelanins. Eumelanins are more photoprotective than pheomelanins, with photoprotection increasing in direct proportion to degree of eumelanin skin pigmentation [4].

The primary purpose of the following review is to provide a quantitative evaluation of agents that have the potential to be used as stimulators of pigmentation in humans. During the course of development, most compounds are tested successively in the following test systems: (i) initial testing is often done in cultured mouse or human melanoma cells; S91 mouse melanoma cells provide a particularly attractive model since they have low basal levels of tyrosinase, and they accumulate melanin intracellularly due to a defect in melanosome export [21]; (ii) testing in melanoma cells is usually followed by testing in cultured normal human epidermal melanocytes; in some cases keratinocyte–melanocyte co-cultures or reconstructed skin models are used; (iii) animal testing is often done using depilated pigmented guinea pigs or pigmented

hairless SkhII mice; and (iv) in some cases human testing has been done. To provide a uniform basis for comparison, this review focuses on summarizing quantitative data from these four test systems (Table 1). Information relating to mechanisms of action is also reviewed, since this often has implications for the efficacy and safety profile of prospective tanning agents. In addition to evaluating stimulators of pigmentation, this review examines topically applied agents that simulate natural pigmentation, since these are presently available to consumers, and they have been shown to have some photoprotective properties. This review is a condensed version of that in Brown et al. [22].

2. Agents that simulate natural pigmentation

2.1. Dihydroxyacetone (DHA)

Cosmetic DHA tanning preparations have been commercially available for nearly half a century [23,24]. Unlike the tanning agents described below (Section 3), DHA does not induce a true-tanning (melanogenic) response. Rather, DHA stains the surface layers of the skin by covalently binding to free amine groups in keratin proteins, followed by a polymerization reaction [24]. Despite this simple staining reaction, DHA tanning has been shown to provide protection against UVA in animals and humans [24–27]. In this regard, it has been shown that application of 15% DHA lotion twice weekly prior to PUVA (oral psoralen plus UVA) treatment for psoriasis, permitted higher UVA treatments with less erythema, and increased the degree and rate of clearing [28]. It should be noted that most over-the-counter DHA products contain only 3 to 5% DHA, but even these have been shown to provide some protection against UVA [24]. Fusaro et al. [27] suggest using DHA tanning in conjunction with sunscreens to reduce UVA exposure, and thereby reduce incidence of malignant melanoma. The combination of DHA tanning and sunscreen usage has been shown to provide good protection against skin eruptions in a variegate porphyria patient [29]. Tanning may last for 5 to 7 days after a single application [24].

2.2. Topical melanins

Similar to DHA, topical application of melanins to skin does not constitute induction of a true-tanning response. However, topically applied melanins have the potential to provide protection both by absorbing and scattering UV radiation, as well as by neutralizing free radicals [24,30,31]. Application of topical synthetic brown melanins at 1% (wt/wt) to SkhII mice exposed to 2 kJ/m² UVB reduced sunburn cell incidence by 72% (3.6-fold) [32]. For humans, topical synthetic brown melanins were

Table 1

Summary of increases of melanogenesis in cultured cells, and photoprotective or pigmentation response in animal and human skin. Values are maximal reported increases. See text and Ref. [22] for detailed explanation and references

	Cultured melanoma cells	Cultured normal human epidermal melanocytes	Animal skin	Human skin
Dihydroxyacetone	Not applicable	Not applicable	Three-fold protection against UVA	Three- to 10-fold protection against UVA
Topical melanins	Not applicable	Not applicable	3.6-Fold protection against UVB	Six-fold increase of sun protection factor
Psoralens plus UVA or SSR	Ten-fold increases of melanin and tyrosinase by 1 μ M 5-MOP plus UVA	Three-fold tyrosinase increase by 1 μ M 5-MOP plus UVA	Three-fold pigmentation increase by 460 μ M 5-MOP plus SSR relative to SSR alone Ten-fold by 513 μ M TMA plus UVA	Three-fold pigmentation increase by 138 μ M 5-MOP plus 2 MED SSR relative to SSR alone
Psoralens in dark	Six-fold melanin increase and 60-fold tyrosinase increase by 50 μ M 5-MOP	None found	None found	Hair follicle melanogenesis stimulated by 100 μ M 5-MOP in DMSO
DMSO	3.3-Fold melanin increase and 15-fold tyrosinase increase by 178 mM	None found	1.6-Fold pigmentation increase by 100% DMSO	No induction
L-Tyrosine and esters	Ten-fold tyrosine increase by 200 μ M	2.3-Fold melanin increase by 1 mM	1.35-Fold pigmentation increase by 48 mM L-tyrosine ester plus SSR relative to SSR alone	1.35-Fold pigmentation increase by 19 mM L-tyrosine ester plus SSR relative to SSR alone
L-Dopa and L-Dopa phosphates	Ten-fold tyrosinase increase by 50 μ M L-Dopa	None found	Ten-fold increase of melanocyte number by 360 μ M in 25% glycerol	None found
Lysosomotropic agents	Three- to four-fold tyrosinase increase by 10 mM NH_4Cl	Ten-fold tyrosinase increase by 8 mM NH_4Cl	None found	None found
Diacylglycerol (OAG)	Three-fold melanin increase by 100 μ M	Seven-fold melanin increase by 100 μ M	Six-fold pigmentation increase by 15 mM	None found
<i>DNA fragments</i> pTpT	Seven-fold melanin increase by 100 μ M pTpT	1.6-Fold melanin increase by 100 μ M pTpT	Pigmentation increased in guinea pig skin by 100 μ M pTpT	None found
Oligos	Six- to eight-fold melanin increase by 100 μ M 5-, 7- and 9-mers	1.6-Fold melanin increase by 100 μ M 9-mer		
MSH and peptide analogs	Nineteen-fold melanin increase and 90-fold tyrosinase increase by 200 nM MSH	1.5-Fold tyrosinase increase and 3.8-fold melanin increase by 10 nM MSH	2.5-Fold pigmentation increase by subcutaneous 4 mg implant	Two-fold pigmentation increase by 0.16 mg/kg injection
IBMX	Twenty-fold melanin and 11-fold tyrosinase increase by 100 μ M	2.2-Fold melanin and seven-fold tyrosinase increase by 100 μ M	None found	None found
NO donors	None found	3.5-Fold melanin increase and five-fold tyrosinase increase by 200 μ M SNP	None found	None found
BMT diols	Twenty-fold melanin increase by 500 μ M 2,2-DM-3-propanyldiol-norbornane	Six-fold tyrosinase increase by 25 μ M 2,2-DM-3-propanyldiol-norbornane	Moderate pigmentation induced by 1 M 2,3- <i>cis/exo</i> -pinanediol	2.3-Fold pigmentation increase by 100 mM 2,2-DM-3-propanyldiol-norbornane

found to provide an SPF of about 6 [33]. Since topical melanins penetrate up to 10 layers of the stratum corneum, they require application only once every 2-3 days for good color retention. However, daily application is recommended [33].

3. Agents that stimulate natural pigmentation

3.1. Psoralens and UVA (PUVA)

Psoralens are usually used to induce pigmentation in

combination with UVA (PUVA). In this case, psoralens have the theoretical possibility to generate DNA crosslinks and monoadducts [34]. However, psoralens have also been shown to stimulate melanogenesis in the dark, presumably with less DNA damage [35]. In cultured cells, only 1 μM is required to stimulate a substantial pigmentation response in the presence of UVR [36], whereas 50 μM is required in the dark [35].

Tanning of skin can be induced either by injection, oral ingestion, or by topical application of psoralens prior to UVA exposure. Injection of guinea pigs with 3 mg/kg 8-methoxypsoralen (8-MOP) followed by 7.6 kJ/m² UVA resulted in a three-fold increase of melanocyte number and induction of moderate pigmentation [37]. Tanning induced in pigmented hairless SkhII mice by topical application of 0.01% (460 μM) 5-MOP in combination with sub-occluded simulated-solar radiation (SSR) for 10 weeks was approximately three-fold greater than with SSR alone [7]. Topical application of 0.117 mg/ml (513 μM) trimethylangelicin (TMA) to SkhII mice in combination with UVA for 13 days resulted in an order of magnitude increase of skin melanin levels as measured by reflectance [8,38]. Melanogenesis was also stimulated in hair follicles by topical application of 100 μM 5-MOP in DMSO to mice maintained in the dark [39]. Daily application of 30 ppm (138 μM) 5-MOP incorporated into a sunscreen to human skin for 2 weeks, followed by treatment with SSR, resulted in three-fold induction of melanin relative to sunscreen alone [12]. Induction of tanning was equivalent to SSR without sunscreen [11–13]. In vitiligo patients, ingestion of 0.6 mg/kg 8-MOP followed by UVA twice per week for 12 weeks resulted in a three-fold increase of melanocytes numbers in depigmented lesions [40].

There is indirect evidence to suggest that induction of melanogenesis by PUVA may be linked to increased expression of p53. Treatment of mouse skin and reconstructed human skin with PUVA has been shown to result in increased p53 expression [41]. Furthermore, a recent study has shown that p53 results in transcriptional activation of both tyrosinase and tyrosinase-related protein 1 [42]. However, additional studies are necessary to demonstrate stimulation of melanogenesis via p53.

Photoactivated psoralens can generate free radicals, and react with lipids and proteins [34]. In vitro, angelicin undergoes a cycloaddition reaction with the central double bond of linolenic acid methyl ester in the presence of UVA [43]. In cultured melanocytes exposed to UVA, 8-MOP forms adducts with phospholipids, and these adducts activate PKC [44]. In pig skin, PUVA has been shown to stimulate adenylate cyclase activity [45]. Psoralens may affect cells kept in the dark by interacting with proteins, nucleic acids and cellular receptors [34]. Induction of tyrosinase by psoralens in the dark was blocked by PKC inhibitors [35], suggesting a mode of action similar to diacylglycerols.

Although PUVA is indicated for treating skin conditions

such as psoriasis, vitiligo, and mycosis fungoides [46], its risks should be weighed against its benefits when considering use as a tanning agent. Treatment of human skin with 5-MOP plus SSR has been shown to be photoprotective against DNA damage otherwise induced by a subsequent dose of SSR [11,12]. However, treatment of SkhII mice with 5-MOP or TMA plus high doses of UVA increased skin tumor occurrence resulting from subsequent exposure to SSR [7,8]. Moreover, psoriasis patients treated with PUVA have a 10-fold increased occurrence of non-melanoma skin cancer [47,48]. In addition, tanning with psoralens plus UVA or sunlight has been associated with severe burn reactions in some individuals [49,50]. Although psoralens stimulate melanogenesis in the dark in laboratory rodents, similar treatment of humans is impractical.

3.2. Dimethylsulfoxide (DMSO)

The use of DMSO as a potential tanning agent is based on the fact that it causes many types of cells to differentiate [51]. One of the markers of melanocyte differentiation is stimulation of melanogenesis. Treatment of melanoma cells with 1.5% (178 mM) DMSO has been shown to result in up to a 3.3-fold increase of melanin content and 15-fold increase of tyrosinase activity [52]. In contrast, one study has shown that DMSO results in a six-fold inhibition of tyrosinase activity induced by the combination of 200 nM MSH and 100 μM IBMX in melanoma cells [53].

DMSO has been shown to result in pigmentation of mouse skin as assessed by subjective rating of histological sections stained with Fontana-Masson [54]. In addition, application of 12.5% (1.5 M) DMSO to mouse ears resulted in a 1.2-fold increase in the density of pigmented cells, while application of 100% (11.9 M) DMSO resulted in a 1.6-fold increase [55]. Moreover, moderate to substantial pigmentation was induced by application of 25 to 100% DMSO to guinea pig skin twice daily for 5 days (D.A. Brown, unpublished). However, treatment of guinea pig skin with 12 to 100% DMSO has been shown to result in an array of inflammatory responses related to the contact urticariogenic and irritant properties of DMSO [56].

Despite the common use of DMSO in topical therapeutic formulations [57–60], there are no reports of induction of pigmentation in humans, even when as much as 10 kg was applied over a period of a year [57]. In fact, application of 50 to 100% DMSO to human skin for 2 to 12 months has been shown to result in a 50% reduction of pigmentation associated with macular and lichen amyloidosis [60]. The therapeutic value of topically applied DMSO appears to be related to its ability to act as an antioxidant and scavenger of free radicals [59,61]. Side effects of topical DMSO therapy have included urticaria (hives), desquamation, and garlic-like breath odor [57,59,60]. Thus, despite its efficacy in cell culture, DMSO does not appear to have potential as a tanning agent in humans.

In addition to its effects as an antioxidant, DMSO has been classified as a polar differentiation agent [51,62]. These types of compounds have been shown to alter cell membrane surface potential by accumulating at the liquid–membrane interface [63]. This may activate membrane-associated signaling pathways including PKC, resulting in cellular differentiation [64]. In this regard, DMSO has also been shown to result in phosphorylation of PKC- ζ in erythroleukemia cells [65]. The effect of DMSO on PKC expression in melanocytes is unknown.

3.3. L-Tyrosine

L-Tyrosine is the precursor compound for the melanogenic pathway. Thus, attempts to induce melanogenesis by L-tyrosine are based on the assumption that the primary substrate for tyrosinase may be limiting. Increasing L-tyrosine from 10 to 200 μM in the media of melanoma cells has been shown to result in a 10-fold increase of tyrosinase activity [66]. In addition, 500 μM L-tyrosine increased the melanin content of normal human epidermal melanocytes co-cultured with keratinocytes approximately 1.6-fold, while 1000 μM resulted in a 2.3-fold increase [67,68].

Despite these positive results in cell culture, application of up to 0.05% (2.7 mM) L-tyrosine to SkhII hairless mice did not induce tanning or enhance UVB-induced tanning [69]. However, lack of response in animal skin using L-tyrosine has been attributed to low treatment concentrations and compound degradation, since 1% (48 mM) L-tyrosine ethylester increased SSR-induced pigmentation 1.35-fold in rat skin [70]. Furthermore, 0.4% (19 mM) L-tyrosine ethylester resulted in a 1.35-fold increase of SSR-induced tanning in human skin [70]. However, no L-tyrosine derivatives or formulations have been demonstrated to induce tanning in animal or human skin without UVR [70,71].

Although L-tyrosine was originally thought to induce melanogenesis by simply increasing substrate for the melanogenic pathway, induction of melanogenesis by L-tyrosine may be related to additional stimulatory mechanisms. L-Tyrosine treatment of hamster melanoma cells resulted in extensive elaboration of melanosomes, an increase in the V_{max} of tyrosinase activity, an increase of tyrosinase protein, and these processes were blocked by inhibitors of protein synthesis [66,72,73]. Furthermore, treatment of hamster melanoma cells with L-tyrosine increased binding of MSH to receptors, and increased tyrosinase induction by MSH [74].

3.4. L-Dihydroxyphenylalanine (L-Dopa) and L-Dopa phosphates

In the first step of the melanogenic pathway, L-tyrosine is converted by tyrosinase to L-Dopa. Subsequently, L-Dopa is converted by tyrosinase to dopaquinone. However,

L-Dopa is not only a substrate for tyrosinase, but it is also an essential cofactor [75]. Treatment of melanoma cells with 50 μM L-Dopa has been shown to increase tyrosinase activity by over 10-fold [66,76]. However, in one instance, treatment of melanoma cells with 200 μM L-Dopa enhanced pheomelanogenesis and decreased eumelanin and tyrosinase in melanosomes, indicating a potential inhibitory effect [77]. In contrast to L-tyrosine, L-Dopa had no effect on the MSH receptor system [74].

L-Dopa is relatively insoluble in aqueous solutions, and is rapidly oxidized to melanin by oxidizing agents. Consequently, L-Dopa phosphates were synthesized since these are water soluble, stable in the presence of oxidizing agents, but hydrolyzed by alkaline phosphatase to yield L-Dopa within cells [78]. Although 10 μM L-Dopa phosphate did not induce tyrosinase in S91 cells, it enhanced the effects of MSH, resulting in a 3.5-fold increase of tyrosinase activity and 2.6-fold increase of melanin content relative to MSH alone [79]. Furthermore, there was three-fold increased binding of MSH to S91 cells [79]. Daily treatment of SkhII mice with 0.01% (360 μM) L-Dopa phosphates in 25% buffered glycerol for 5 weeks resulted in a 10-fold increase of detectable melanocytes [80]. Furthermore, 0.01% L-Dopa phosphates resulted in a two-fold enhancement of detectable melanocytes induced by thrice weekly treatment with 1.34 kJ/m^2 UVB plus 1.46 kJ/m^2 UVA [80]. Unphosphorylated L-Dopa was ineffective in these studies.

The major concern with regards to use of L-Dopa and L-Dopa phosphates is related to potential toxicity. Although the oxidation products of L-Dopa are toxic to cells, during melanogenesis melanocytes are shielded from this toxicity by virtue of the fact that metabolites of melanin synthesis are partitioned into melanosomes [81]. Release of L-Dopa or L-Dopa phosphate oxidation products from melanosomes or oxidation of L-Dopa outside of melanosomes has the potential to result in significant toxic effects [79,81,82]. Since L-Dopa is used for treating Parkinson's disease, the undesirable side effects on humans, including motor fluctuations and psychiatric complications, are well known [83]. The effects of chronic administration of L-Dopa phosphates have not been documented.

3.5. Lysosomotropic agents

There is evidence to indicate that melanosomes from Caucasian and Black skin contain similar levels of tyrosinase, but that tyrosinase activity is inhibited in Caucasian melanosomes due to their more acidic pH [84,85]. Treatment of Caucasian melanocytes with the lysosomotropic agent ammonium chloride increases tyrosinase activity by increasing the melanosomal pH [85]. Ammonium chloride (8–10 mM) has been shown to increase in situ tyrosinase activity of S91 mouse melanoma cells up to four-fold [86,87], and that of Caucasian melanocytes 10-fold [85]. This increase of tyrosinase activity in Caucasian

melanocytes may be related to an unknown modification of tyrosinase since it occurred without increases of tyrosinase protein [85]. There are no published studies on the effects of lysosomotropic agents on induction of melanogenesis in animals or humans.

3.6. Diacylglycerols (DAG)

Similar to UVR, diacylglycerols activate the PKC signaling pathway within cells. Stimulation of the PKC pathway has been shown to be associated with both proliferation and differentiation of melanocytes [88,89]. Treatment with 100 μ M 1-oleyl 2-acetyl glycerol (OAG), a synthetic DAG, has been shown to increase the melanin content three-fold in melanoma cells and seven-fold in normal human epidermal melanocytes (NHEM) [88]. Treatment of reconstructed human epidermis with 200 μ M OAG resulted in a 2.5-fold increase of melanin content [90]. In contrast to these results, one study has shown that 100 μ M OAG inhibits tyrosinase activity and melanin production in cultured B16 mouse melanoma cells [91]. Application of 15 mM OAG resulted in a six-fold increase of melanin deposition in skin of SkhII mice [92]. For guinea pigs, application of 25 mg/ml (63 mM) OAG in propylene glycol resulted in a 3.7-fold increase of melanin deposition [93]. OAG treatment acted synergistically with UVR to induce melanogenesis in both cultured cells and animal skin [88,92].

Various stimuli including UVR are known to increase cellular DAG levels and activate PKC [94–96]. Since DAG is an activator of PKC, it is thought that DAG and UVR might activate melanogenesis by a PKC-dependent pathway [97]. Support for a role of PKC in induction of melanogenesis is provided by studies showing that: inhibitors of PKC block induction of melanogenesis by OAG in NHEM [98], PKC depletion blocks stimulation of melanogenesis by α -MSH in S91 cells [99], and the PKC- δ activator bistratene A stimulates melanogenesis in a human melanoma cell line [100]. Moreover, Park et al. [101] have demonstrated that PKC- β is closely associated with tyrosinase on the surface of melanosomes, and that PKC- β activates tyrosinase by phosphorylating it at cytoplasmic serine residues [101]. However, Carsberg et al. [102] found that down-regulation of PKC with 12-*O*-tetradecanoylphorbol-13-acetate (TPA), or treatment of cells with a PKC inhibitor did not block the ability of either OAG or UVR to induce melanogenesis. These authors conclude that OAG and UVR induce melanogenesis by PKC-independent pathways. Thus, the mechanism by which OAG induces melanogenesis remains unsettled.

Although DAGs appear to have considerable efficacy with regards to induction of melanogenesis, there are safety issues with regards to using DAGs as pigmentation agents. There has been some suggestion that topical treatment with OAG and other DAGs may result in

inflammation [93]. Furthermore, activation of PKC by UVA has been associated with tumor promotion [94,96]. Some DAGs activate PKC, stimulate epidermal hyperplasia, and are tumor promoters in mouse skin [103–105]. Furthermore, OAG has been shown to result in transformation in cell culture carcinogenesis models [106,107].

3.7. pTpT and DNA fragments

The use of pTpT (thymidine dinucleotides) and DNA fragments to induce melanogenesis is based on the consideration that these are generated during the repair process following UVR, and as such they may provide a signal to stimulate a photoprotective response [108]. Treatment with 100 μ M pTpT has been shown to increase the melanin content seven-fold in melanoma cells and 1.6-fold in NHEM [109,110]. Application of 100 or 300 μ M pTpT in full strength propylene glycol or a mixture of propylene glycol and DMSO (75%:25%) twice per day for 5 days resulted in a marked pigmentation response in depilated guinea pig skin [109]. Additional DNA fragments including 5-, 7- and 9-mer oligonucleotides have been shown to stimulate melanin production six- to eight-fold in S91 cells [108,111], and a 9-mer has been shown to stimulate melanin production 1.6-fold in human melanocytes [111]. Although pTpT and DNA fragments have been shown to increase tyrosinase expression in cultured cells [108–111], there is a paucity of studies examining effects on tyrosinase activity.

In addition to its effects on melanogenesis, pTpT has been reported to increase the binding of MSH to its cell surface receptor, activate p53, increase p53 binding to its DNA consensus sequence, and stimulate repair of UV-induced photoproducts [112–115]. Furthermore, pTpT stimulates expression of p21, proliferating-cell nuclear antigen, GADD45, ERCC3, and XPA protein [113–115]. These results have led to the suggestion that pTpT stimulates a eukaryotic “SOS” response, but without the damage to DNA incurred by UV exposure [108,113,116]. In fact, treatment of depilated guinea pigs with 100 μ M pTpT in propylene glycol:DMSO (75%:25%) for 1 or 2 weeks (total 10 treatments) resulted in a tanning response that was photoprotective against epidermal damage and necrosis induced by 6 MED (minimal erythemal dose) UVB [116]. There are no reported results of the tanning effect of pTpT on human skin.

It has been suggested that the basis for induction of melanogenesis by pTpT and DNA fragments is related to the removal of pyrimidine dimers during DNA repair following UVR exposure [112,116]. In this regard, it has been demonstrated that stimulation of DNA repair by treatment of cells with the DNA repair enzyme T4 Endonuclease V stimulates melanogenesis in S91 cells [117]. In mammalian cells, excision of pyrimidine dimers is mediated by the nucleotide excision repair (NER) system, which removes patches of \sim 30 nucleotides that

encompass pyrimidine dimers. Therefore, pTpT and 5- to 9-mer DNA fragments could occur as degradation products of DNA fragments excised during repair. The mechanism by which these degradation products might induce melanogenesis is unknown, but may be related to induction of p53, since p53 has been shown to transcriptionally activate tyrosinase and tyrosinase-related protein 1 [42].

Thymidine dinucleotides have been shown to inhibit induction of contact hypersensitivity in mouse skin, and activate the expression of immunosuppressive TNF- α in skin cells [118]. Inhibition of the contact hypersensitivity response in human skin by topical application of 100 μ M pTpT was equivalent to that induced by 200 J/m² UVB. This response was not induced by pApdA or a propylene glycol vehicle control. The fact that pTpT induces TNF- α and inhibits contact hypersensitivity when applied to human skin indicates that it has immunosuppressive properties. In the context of utilizing pTpT as a tanning agent, it should be noted that the immunosuppressive effects of UV have been associated with decreased immune surveillance and resultant increased tumor development in animal and human skin [119]. Therefore, based on results with pTpT, it would be prudent to examine the immunosuppressive effects of any substance that might be contemplated for use as an artificial tanning agent.

3.8. Melanocyte stimulating hormone (MSH) and peptide analogs

MSH and analogs have been shown to induce pigmentation both when applied topically to animal skin, or injected subcutaneously. Administration of 100 nM MSH to S91 cells increased tyrosinase activity approximately three-fold, while 100 nM of the MSH analog [Nle⁴,D-Phe⁷]- α -MSH increased tyrosinase nearly seven-fold [120]. The MSH analog is less subject to degradation than MSH and has a greater affinity for the MSH receptor. In studies with pigmented hairless hr/hr BOM mice or pigmented guinea pigs, topical application of 1 mg/ml (0.4 mM) β -MSH in buffered 25% glycerol failed to induce pigmentation [121]. However, co-treatment with 1 mg/ml β -MSH resulted in a 5.5-fold enhancement of pigmentation induced in guinea pig skin by 30 kJ/m² UVB administered over a 2 week period [121]. Similar results were seen in hairless mice [121]. When applied topically in a polyethylene glycol ointment, MSH stimulated hair follicular melanogenesis at 10 nM, and MSH analogs stimulated this process at concentrations as low as 10 fM [122]. Coloration of hair occurred at the site of application, and distant sites, demonstrating that topical application results in systemic delivery [122]. Subcutaneous administration of controlled-release implants containing 4 mg [Nle⁴,D-Phe⁷]- α -MSH in hairless and haired pigmented guinea pigs produced a pigmentation response that reached a peak after 1 month, and was maintained for an additional 3 months [123]. In the case of hairless guinea pigs, there was a 2.5-fold

increase of eumelanin content as measured by HPLC analysis of potassium permanganate treated skin biopsies [123]. Haired guinea pigs showed a distinct darkening of coat color, indicating that melanogenesis was stimulated in hair follicles as well as skin.

MSH and analogs induce tanning in human skin. Ten subcutaneous injections of [Nle⁴,D-Phe⁷]- α -MSH at 0.08 mg/kg of body weight over 12 days showed significant skin darkening that peaked during weeks 3 through 5 of the study, and faded somewhat by week 9 [124]. Skin darkening was greatest in areas previously tanned such as the face and neck, with no darkening of the trunk or buttocks [124]. Results were similar in a subsequent study using a more potent synthetic MSH analog (Melanotan-II) injected at 0.01 mg/kg body weight with 0.005 mg/kg dose escalations over 10 injections [125]. Quantitative HPLC analysis of skin biopsies from subjects injected with 0.16 mg/kg [Nle⁴,D-Phe⁷]- α -MSH for 10 days showed a 1.5-fold increase of eumelanin in forehead skin, and a two-fold increase in forearm skin [126]. Side effects of [Nle⁴,D-Phe⁷]- α -MSH injections include erythematous flushing, yawning, lethargy, nausea, and spontaneous penile erections [125,126]. Given these side effects, the unevenness of tanning, and the inconvenience of administration by injection, MSH analogs appear to have limited potential as tanning agents.

It has been suggested that MSH stimulates melanogenesis via the cAMP signaling pathway. Stimulation of a receptor complex by MSH increases cAMP, which is followed by elevations of tyrosinase activity in S91 cells [127]. Furthermore, cAMP analogs stimulate melanogenesis in S91 cells, suggesting a link to induction of melanogenesis by MSH [127]. In addition, treatment of S91 cells with MSH has been shown to result in a transitory increase of cAMP levels, followed by down-regulation of the MSH receptor–adenylate cyclase complex [128]. While these previous studies strongly implicate cAMP signaling in induction of melanogenesis by MSH, it has been shown that an inhibitor of adenylate cyclase (2',5'-dideoxyadenosine) enhanced the melanogenic response to 100 nM MSH in S91 melanoma cells [129]. Thus, the role of cAMP in MSH signaling is unclear.

Moreover, while there is evidence suggesting that UVB induces melanogenesis via a MSH-receptor mediated pathway [121,130,131], UVR does not appear to induce melanogenesis via the cAMP pathway [132]. In this regard, a recent study has shown that as little as 1 pM α -MSH results in a four-fold increase of nitric oxide synthesis in B16 mouse melanoma cells, and a two-fold increase in FM55 human melanoma cells [133]. As discussed below, nitric oxide has been mechanistically linked to induction of both melanogenesis and erythema by UVR. Thus, the induction of nitric oxide by MSH may play a role in stimulation of melanogenesis. Furthermore, induction of nitric oxide by α -MSH may explain some of its side effects, including spontaneous flushing and erec-

tions, since these are well known to be mediated by the nitric oxide/cGMP signal pathway [134,135].

3.9. 3-Isobutyl-1-methylxanthine (IBMX)

Methylxanthines including caffeine, theophylline and IBMX are phosphodiesterase inhibitors [136,137]. IBMX is a well-tested and potent stimulator of melanogenesis. Consequently, IBMX is often used as a positive control in melanogenesis studies [109,110,138]. Treatment of melanoma cells with 100 μ M IBMX has been shown to increase melanin content and tyrosinase activity by an order of magnitude [109–111,120,136]. Treatment of cultured NHEM with 100 μ M IBMX stimulated cellular proliferation 1.8-fold, and tyrosinase activity up to seven-fold [89]. In human reconstructed skin models, 100 μ M IBMX increased melanin content 2.2-fold [90]. Furthermore, treatment of human reconstructed skin with 50 μ M IBMX increased melanosome deposition in keratinocytes and nuclear capping, similar to SSR [139]. Despite the reported efficacy of IBMX in cell culture, there are no published reports of efficacy when applied to animal or human skin.

It is generally believed that IBMX and other methylxanthines stimulate melanogenesis by inhibiting phosphodiesterases and thereby increasing levels of cAMP [87,129,140]. Furthermore, there has been clear evidence that dibutyryl cAMP increases tyrosinase activity and mRNA expression in S91 cells [137]. However, although treatment of S91 cells with IBMX increases cAMP levels, it has been shown that an inhibitor of adenylate cyclase (2',5'-dideoxyadenosine) enhances the melanogenic response to IBMX [129]. Thus, similar to MSH, the role of cAMP in IBMX signaling is unclear.

In this regard, it should be noted that IBMX is an inhibitor of both cAMP and cGMP phosphodiesterases [141–144]. Consequently, both cAMP and cGMP may be increased by IBMX treatment [144]. Furthermore, in SCC-13 squamous carcinoma cells, both dibutyryl cGMP and IBMX increased nitric oxide (NO) production by a putative positive feedback loop [145], indicating a potential role for NO in the effects of IBMX. Since the NO/cGMP pathway is now known to stimulate melanogenesis, the role of cGMP phosphodiesterase inhibition by IBMX in relation to melanogenesis warrants further study.

When applied to skin of mice, IBMX has been shown both to promote and inhibit tumorigenesis. When skin tumors were initiated with DMBA (dimethyl-benz[*a*]anthracene) followed by treatment with IBMX prior to promotion with TPA, IBMX had an inhibitory effect [136]. However, when skin was treated with IBMX prior to initiation with DMBA, tumor incidence increased [146]. In contrast, cAMP inhibited both tumor initiation and promotion. In vitro experiments using isolated skin cells showed that cAMP stimulated, and IBMX inhibited, DMBA-induced unscheduled DNA synthesis, indicating that IBMX

may enhance tumor initiation by inhibiting DNA repair [146].

3.10. Nitric oxide (NO) donors

Despite the number of prospective tanning agents that claim to induce melanogenesis via the PKC or cAMP/PKA pathways, it has been shown that UVR does not induce melanogenesis by either of these pathways [102,132]. Rather, UVR appears to stimulate melanogenesis via the nitric oxide/cyclic guanosine monophosphate/protein kinase G (NO/cGMP/PKG) pathway, with NO generated from both irradiated melanocytes and keratinocytes [147,148]. Nitric oxide so generated stimulates not only melanogenesis, but also erythema, since NO is a vasodilator [149]. Additional sources of NO in the skin include dermal fibroblasts, vascular cells and inflammatory cells [149–151]. Nitric oxide is produced in cells when L-arginine is metabolized to L-citrulline by nitric oxide synthase (NOS).

The effect of NO donors on induction of melanogenesis was first suggested by Romero-Graillet et al. [147]. Treatment of cultured NHEM with 200 μ M sodium nitroprusside (SNP) for 4 days resulted in three- to five-fold increases of tyrosinase activity, and two- to 3.5-fold increases of melanin synthesis [147,148]. Since NO is known to stimulate guanylate cyclase activity, they then treated NHEM with the nonhydrolyzable cGMP analog 8-bromo-cGMP (8-br-cGMP). Treatment with 5 mM 8-br-cGMP resulted in a two-fold increase of tyrosinase activity and melanin synthesis, while 10 mM 8-br-cGMP resulted in a four- to five-fold increase of these parameters [147]. Treatment with inhibitors of guanylate cyclase and PKG blocked induction of melanogenesis by NO donors, demonstrating that these induce melanogenesis by the NO/cGMP/PKG pathway [147,152]. Furthermore, Romero-Graillet et al. [147] demonstrated that UVB irradiation of NHEM results in production of cGMP, and that induction of melanogenesis by UVB is blocked by inhibitors of NOS, guanylate cyclase, and PKG. In a subsequent study, they demonstrated that normal human epidermal keratinocytes (NHEK) also synthesize NO in response to both UVA and UVB irradiation, and that NO scavengers inhibit induction of NHEM tyrosinase activity in NHEM/NHEK co-cultures [148]. These results demonstrate that, following UVB irradiation, NO induces melanogenesis by both autocrine and paracrine mechanisms.

As of this writing, there are no published studies showing the effect of NO donors on induction of pigmentation in animals or humans. However, inhibitors of NOS have been shown to inhibit induction of both erythema and pigmentation by UVB in guinea pig skin [153].

Although NO may have beneficial effects in skin, including mediation of wound healing [151], it may be damaging when it reacts with superoxide to form highly

reactive peroxy-nitrite anion [151,154]. Peroxy-nitrite induces lipid and protein peroxidation, tyrosine nitration, and has been implicated in a number of disease processes [151,154]. Peroxy-nitrite damages DNA with a potency that is at least 100-fold greater than NO, implicating peroxy-nitrite as the penultimate agent in NO-induced DNA damage [155]. Elevated formation of NO and peroxy-nitrite in squamous cell carcinoma relative to normal keratinocytes following UVA irradiation has been related to a poor prognosis [145].

Paradoxically, though, stimulation of the NO/cGMP/PKG pathway may be useful for treating several different types of cancers [156–159]. Since NO regulates vascular tone and is a major neurotransmitter in the autonomic nervous system, its dysregulation may result in diverse pathophysiological conditions [160]. Given these diverse effects, the use of NO releasing or stimulating agents to induce tanning must be approached with some caution. Since the half-life of NO is only 3 to 5 s [160], it is unlikely that NO generated in the skin would have significant effects in other tissues. However, agents that are transported transdermally (e.g., topically applied MSH as discussed in Section 3.8) have the potential to release or induce NO production in other tissues.

3.11. Bicyclic monoterpene (BMT) diols

The discovery that diols induce melanogenesis originated with the observation that 1,2-propanediol (propylene glycol), a commonly used vehicle for topical administration of melanogenic agents to animal skin, was itself melanogenic [138]. Following this initial observation, a series of aliphatic diols of increasing complexity were examined, the most potent of which was 3,3-dimethyl-1,2-butanediol [138]. Several monocyclic diols (e.g., 1,2-*cis*-pentanediol) were then examined, but they were only slightly more potent than 3,3-dimethyl-1,2-butanediol [138]. Subsequently, to investigate compounds with a more rigid structure and stable presentation of functional groups [161], BMT diols were examined.

BMT diols have been found to be efficacious both when applied to cultured cells and skin. Treatment of melanoma cells with 500 μM 2,2-dimethyl-3-propanyldiol-norbornane has been shown to result in 20-fold increases of melanin [22]. Treatment of co-cultures of NHEK and NHEM from a black donor with 25 μM 2,2-DM-3-propanyldiol-norbornane resulted in a six-fold increase of tyrosinase activity as measured by the L-Dopa reaction [22]. When applied to guinea pig skin, BMT diols resulted in induction of a significant pigmentation response, supranuclear capping as indicated by Fontana-Masson staining of biopsy samples, and thickening of the epidermis similar to that occurring as a result of UVB irradiation [138,159].

BMT diols have also shown some efficacy in a human pilot study, with improved tanning when α -hydroxy acids

or retinoids were included in the formulation [22]. Similarly, treatment with α -hydroxy acids or retinoids has been shown to increase UVR-induced tanning in humans, indicating a direct effect on the pigmentation response [162–166]. The mechanisms by which α -hydroxy acids and retinoids enhance tanning by UVR or BMT diols are unknown, but may be related to increased epidermal proliferation, and increased dispersion of melanosomes [167,168]. In addition, one study has shown that retinoids up-regulate MSH receptor expression three- to four-fold in S91 melanoma cells [169], but it is not known if this occurs in human skin.

Similar to the effect of UVR in human melanocytes and keratinocytes [147,148], BMT diols induce melanogenesis by the NO/cGMP/PKG pathway in S91 melanoma cells [159]. As discussed above, although melanogenesis can be induced by agents that stimulate the PKC and PKA signaling pathways, UVR does not stimulate melanogenesis by these pathways [102,132]. Nor do BMT diols stimulate melanogenesis by the PKC or PKA pathways, since inhibitors of these pathways actually enhanced the effect of BMT diols on melanogenic response [159]. The fact that BMT diols induce melanogenesis by the same pathway as UVR may have implications for efficacy.

Monocyclic and bicyclic monoterpenes are abundant in plants and foods, and are rapidly metabolized and excreted by mammals [170]. All except one monoterpene tested were nonmutagenic in the Ames test [171], and 5 mM 5-norbornene-2,2-dimethanol is not mutagenic in either shuttle vector or HPRT mutation assays [22]. Thus, BMT diols are likely to have a good safety profile. However, further studies are warranted to determine if BMT diols have any undesirable side effects due to stimulation of NO production.

4. Summary and prospectus

This review shows that there are a number of agents in development to increase pigmentation of skin. Those that stimulate pigmentation are already on the market. Other agents stimulate melanogenesis in cultured cells and animal skin. Results in Table 1 indicate that efficacy in melanoma cells is often predictive of efficacy in cultured NHEM and animal or human skin. However, much greater concentrations are often required to induce melanogenesis in living skin. One possible reason for this is that there is a 100–1000 dilution factor from the surface of the skin to the lower layers of the epidermis [172].

Safety should be the major concern when using some of these agents as potential tanning agents. However, benefits must also be considered. Since natural pigmentation is associated with greatly reduced skin cancer risk, the use of pigmentation stimulators before sun exposure has the potential to reduce cancer risk.

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