

Review Article

The melanogenesis and mechanisms of skin-lightening agents – existing and new approaches

J. M. Gillbro and M. J. Olsson

Oriflame Cosmetics Skin Research Institute, SE-101 39 Stockholm, Sweden

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Keywords: adrenergic, melanogenesis, skin lightening, tyrosinase, vitiligo**Synopsis**

Skin-lightening products are commercially available for cosmetic purposes to obtain lighter skin complexion. Clinically, they are also used for treatment of hyperpigmentary disorders such as melasma, café au lait spot and solar lentigo. All of these target naturally melanin production, and many of the commonly used agents are known as competitive inhibitors of tyrosinase, one of the key enzymes in melanogenesis. In this review, we present an overview of commonly used skin-whitening ingredients that are commercialized, but we also hypothesize on other mechanisms that could be important targets to control skin pigmentation such as for example regulation of the adrenergic and glutaminergic signalling and also control of tetrahydrobiopterins in the human skin.

Résumé

Les produits éclaircissants sont disponibles dans le commerce pour des buts cosmétiques afin d'obtenir un teint plus clair. Ils sont également utilisés en clinique, pour le traitement de troubles hyper pigmentaires comme le melasma, les taches café au lait et le lentigo solaire. Tous ces produits ont pour cible la production naturelle de mélanine et beaucoup de ceux généralement utilisés sont reconnus comme des inhibiteurs compétitifs de la tyrosinase, une des enzymes clés de la mélanogénèse. Dans cette revue, nous présentons une vue d'ensemble des ingrédients généralement utilisés et commercialisés comme blanchissant cutanés mais nous formulons aussi l'hypothèse que d'autres mécanismes pourraient être des cibles importantes pour contrôler la pigmentation de la peau comme par exemple la régulation du signal adrénérgique et glutaminérgique ou le contrôle des tétrahydrobioptéridines dans la peau humaine.

Introduction**Melanogenesis**

Human skin colour stems from in the outermost layer of the skin, the epidermis where the pigment-producing cells melanocytes are localized to produce melanin. Upon exposure of the skin to UV radiation, melanogenesis is enhanced by the activation of the key

enzyme of melanogenesis, tyrosinase. Tyrosinase is a glycoprotein located in the membrane of the melanosome, a minifactorial vesicle inside the melanocyte (Fig. 1). It has an inner melanosomal domain that contains the catalytic region (approximately 90% of the protein), followed by a short transmembrane domain and a cytoplasmic domain composed of approximately 30 amino acids [1]. Histidine residues are present in the inner (catalytic) portion of tyrosinase and bind copper ions that are required for tyrosinase activity [2]. Melanogenesis takes place in the melanosomes. Two types of melanin are synthesized within melanosomes: eumelanin and pheomelanin [3]. Eumelanin is a dark brown-black insoluble polymer, whereas pheomelanin is a light red-yellow sulphur-containing soluble polymer [3].

Tyrosinase catalyses the first two steps of melanin production: the hydroxylation of L-tyrosine to L-dihydroxyphenylalanine (L-DOPA) and the subsequent oxidation of this *o*-diphenol to the corresponding quinone, L-dopaquinone [4–7]. Even though L-tyrosine is the building stone for melanin, it can only be transported into the melanosome by facilitated diffusion [8, 9]. In this context, it is noteworthy that the concentration of L-tyrosine for melanogenesis depends on the conversion of the essential amino acid L-phenylalanine by intracellular phenylalanine hydroxylase (PAH) activity and in contrast to L-tyrosine, L-phenylalanine is actively transported through the melanosomal membrane to ensure high content of L-tyrosine inside this organelle. The importance of

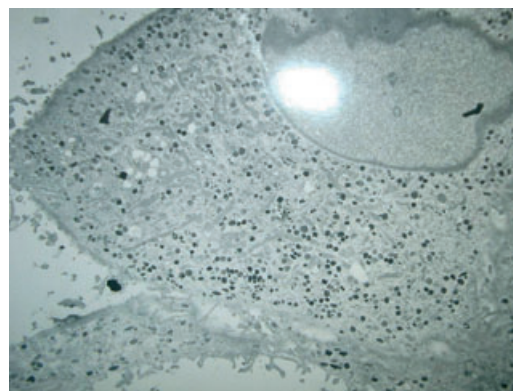


Figure 1 Electron microscopy picture of a DOPA-stained melanocyte. Note the abundant numbers of dark and well-defined melanosomes.

Correspondence: Johanna M. Gillbro, Nordenflychtsvägen 62, Stockholm, Sweden. Tel.: +46 858632300; fax: +46 8560912; e-mail: johanna.gillbro@oriflame.com

L-phenylalanine for melanogenesis is demonstrated in the skin phototypes I–VI where epidermal PAH activities are correlated linearly [10].

Following the formation of dopaquinone, the melanin pathway is divided into synthesis of the black-brownish eumelanin and red-yellow pheomelanin [11] where there is a spontaneous conversion to leucodopachrome and dopachrome. In the eumelanin pathway, dopachrome is either spontaneously converted to 5,6-dihydroxyindole or is enzymatically converted to 5,6-dihydroxyindole-2-carboxylic acid via enzymatic conversion by dopachrome tautomerase (DCT), also referred to as tyrosinase-related protein-2 (TRP-2). There are two tyrosinase-related proteins, TRP-1 and TRP-2, which are structurally related to tyrosinase and share approximately 40% amino acid homology, suggesting that they originated from a common ancestral gene [12–14]. TRP-1 and TRP-2 reside within the melanosomes and, like tyrosinase, span the melanosomal membrane [15]. It has been suggested that TRP-1 increases the ratio of eumelanin to pheomelanin [16, 17]. In addition, they have been demonstrated to increase tyrosinase stability [18, 19]. However, the role of TRP-1 and TRP-2 is not totally clarified yet, and it is also not clear whether other enzymes also play important roles in the eumelanogenic pathway.

Finally, the polymerization of indoles and quinones leads to eumelanin formation [20]. The pheomelanin pathway branches from the eumelanin pathway at the L-dopaquinone step and is dependent on the presence of cysteine which is actively transported through the melanosomal membrane. Cysteine reacts with L-dopaquinone to form cysteinyl-dopa [20]. The latter is then converted to quinoleimine, alanine-hydroxyl dihydrobenzothiazine and polymerizes to pheomelanin.

Tyrosinase can also be indirectly activated by tyrosine hydroxylase isoenzyme 1 (TH1) as it has been shown to be present in melanosomes and catalyzes L-dopa synthesis [21]. In turn, L-dopa can act as a cofactor for tyrosinase [22].

Redox conditions in the melanosomes are crucial for the balance between the production of eumelanins and pheomelanins. The formation of eu- or pheomelanin is directly determined by reduced glutathione (GSH) (high GSH for eumelanin and low for pheomelanin). Therefore, the expression and functional activity of antioxidant enzymes such as catalase, glutathione peroxidase, glutathione reductase and thioredoxin reductase likely modify the melanogenic pathway [23] (Fig. 2).

Also, melanin itself has an important role in oxidative homeostasis in the skin. Eumelanin has an ability to both scavenge and quench both oxygen- and carbon-derived free radicals [24, 25]. Pheomelanin do not have the same properties and can even be a source for free radical production when UV irradiated. Besides quenching free-radicals and acting as a physical barrier against UV radiation, the melanin polymer through its negatively charged properties has the ability to bind amines and heavy metals [26].

Melanogenic regulatory proteins

The discovery about 10 years ago of the gene encoding the basic helix–loop–helix leucine zipper microphthalmia-associated transcription factor gene (MITF) [27, 28], provided a major impetus to the study of transcription regulation in the melanocyte lineage. Indeed, MITF appears to be at the heart of a regulatory network of transcription factors and signalling pathways that control the survival, proliferation and differentiation of melanoblasts and melanocytes (reviewed in [29]). Not only the melanocyte development is affected by this protein but also pigmentation via its transcriptional regulatory effect on tyrosinase, TRP-1 and TRP-2 [30]. MITF was shown to be a key transcription factor for Rab27A [31], a protein important for melanosome transport. Therefore, MITF plays a central role in melanin synthesis, as well as melanosome biogenesis and transport.

Paracrine melanogenic stimulators

There are number of paracrine stimulators of melanogenesis such as proopiomelanocortin (POMC)-derived peptides (α -MSH, β -MSH, ACTH) [32]. These melanotrophic hormones were discovered in the early 1950s by Dr. Aaron B. Lerner [33–35]. POMC expression in keratinocytes is induced by UV [36]. The pivotal effect of these hormones on melanogenesis has been demonstrated *in vivo* where systemic administration of α -MSH, β -MSH, or ACTH increases skin pigmentation predominantly in sun-exposed areas [37, 38]. The POMC peptides exert its effect through a cyclic adenosine 3',5'-monophosphate (cAMP)-dependent mechanism when binding to the Gs-protein-coupled receptor melanocortin receptor 1 (MC1R) [39–42]. This intracellular second messenger is well known to regulate melanogenesis. Stimulation of specific Gs-protein-coupled

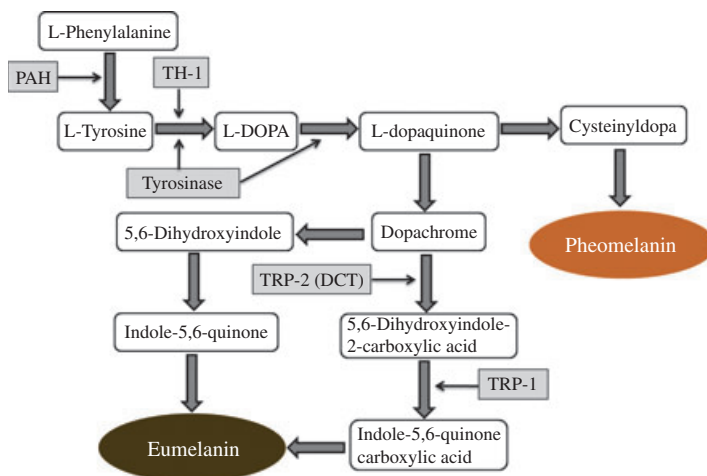


Figure 2 Melanin synthetic pathway. Melanin synthesis begins with catalysation of the substrates L-phenylalanine and L-tyrosine to produce L-DOPA via phenylalanine hydroxylase (PAH), tyrosinase and partly tyrosinase hydroxylase 1 (TH-1). The pathways are then divided into eumelanogenesis or pheomelanogenesis. The other melanogenic enzymes are TRP-2 (DCT) and TRP-1 for eumelanogenesis. No specific enzymes have been found that are involved in pheomelanogenesis so far.

receptors leads to the activation of adenylyl cyclase (AC). AC produces cAMP which consequently stimulates the melanogenic pathway [39–42]. This involves the activation of protein kinase A (PKA), which then phosphorylates enzymes, ion channels and several regulatory proteins eventually leading to a change in gene expression. Regulation of transcriptional activity by activated PKA involves phosphorylation of cAMP-responsive element-binding protein (CREB) and activation of microphthalmia-associated transcription factor (MITF) [43, 44]. In turn, MITF efficiently activates the melanogenic enzyme genes, such as tyrosinase and TRP-1/TRP-2 in cultured cells [45, 46] (Fig. 3).

It was recently discovered that α -MSH can increase melanin synthesis by a mechanism independent of MC1R by binding to 6(R)-L-erythro-5,6,7,8-tetrahydrobiopterin (6BH₄), a competitive inhibitor of tyrosinase, and release the inhibitory effect on tyrosinase activity [47].

Even though the POMC peptides have important effect on human skin colour, there are other paracrine factors that are of pivotal importance for skin pigmentation such as endothelin-1, stem cell factor, prostaglandins and catecholamines to mention some [48–52].

Skin-lightening ingredients

As of increasing focus on skin appearance, many cosmetic and pharmaceutical companies are focusing on research that will alter skin pigmentation.

There are today many known substances that can reduce the level of pigmentation in the skin. Many of these actives have a tyrosinase-inhibiting effect leading to reduced total melanin produc-

tion. Some of the tyrosinase inhibitors used today is for example kojic acid, arbutin and different kinds of vegetal or herb extracts. There are also molecules known to have an effect on the transfer of melanin from melanocytes to keratinocytes, leading to an overall lighter skin colour such as nicotinamide and soyabean. Substances that increase the desquamation of the skin are also commonly used to remove excessive melanin content within the skin, for instance retinoic acid.

In this article, we present a review of several important depigmenting and lightening agents reported in the literature for use in skin-lightening products. Also, new hypothesis for mechanistic skin-lightening targets are proposed.

Skin-lightening activity by tyrosinase inhibition

The most common target for skin-lightening activities is tyrosinase inhibition and below some of the most commonly used ones are reviewed.

Quinone-related compounds

Hydroquinone (1,4-dihydroxybenzene) has been the conventional standard for treating hyperpigmentation for more than 40 years [53–55].

The compound can be found in tea, wheat, berries, beer and coffee. Hydroquinone interacts with tyrosinase by binding histidines at the active site of the enzyme resulting in reduction in skin pigmentation in general, in melanosis but also unaffected skin of vitiligo patients to reduce overall pigmentation [56]. Additionally, hydroquinone induced generation of reactive oxygen species, and

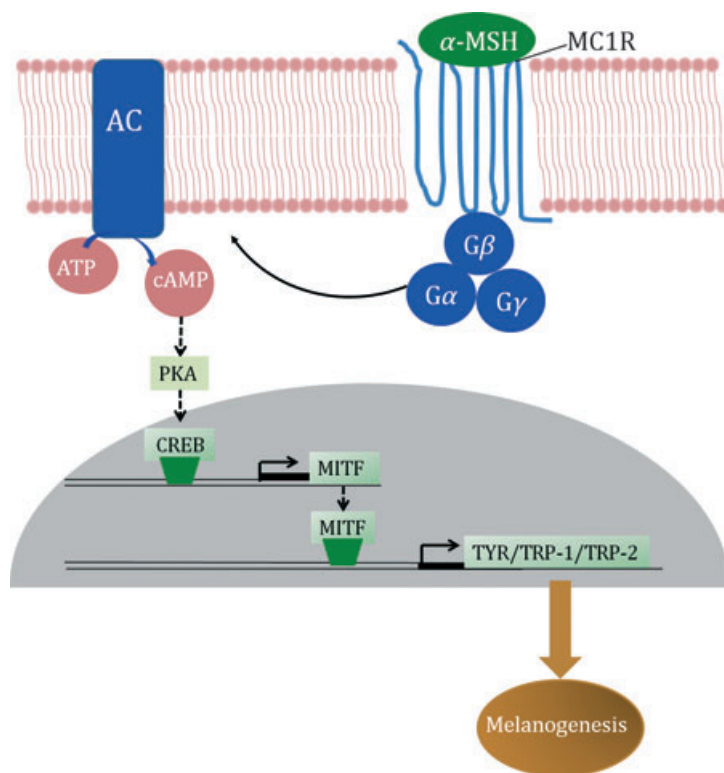


Figure 3 The melanocortin signalling pathway. α -MSH binds to and activates the Gs protein-coupled MC1R. The G_s family of G proteins (including G α , G β and G γ) transmits signals from MC1R to AC which, in turn, catalyses the conversion of cytoplasmic ATP to cAMP. Increased levels of cAMP act as a second messenger to activate PKA, which, upon activation, translocates to the nucleus where it phosphorylates the CREB family of transcription factors. Phosphorylated CREBs then induce the expression of genes containing CRE (cAMP-responsive elements) consensus sequences in their promoters, such as the transcription factor MITF. The transcription factor MITF binds to the promoter of the pigmentary genes tyrosinase TRP-1 and TRP-2 (DCT).

quinones leads to the oxidative damage of membrane lipids and proteins such as tyrosinase.

Hydroquinone is also thought to inhibit pigmentation by depleting glutathione, reducing DNA and RNA synthesis with concomitant melanosome degradation and melanocyte damage [57–61].

However, the golden days of hydroquinone seem to have come to an end as this potent skin-lightening agent can lead to permanent loss of melanocytes because of its oxidative damage of membrane lipids leading to irreversible loss of inherited skin colour [53]. In addition, it was recognized that this substance is transported rapidly from the epidermis into the vascular system and is detoxified within the liver into inert compounds [62, 63]. Because of the risks of side effects such as permanent depigmentation and exogenous ochronosis following long-term use, hydroquinone has been banned by the European Committee (24th Dir 2000/6/EC).

Another commonly used quinone used for skin-lightening purposes is arbutin, which is a derivative of hydroquinone (hydroquinone-*O*- β -D-glucopyranoside) and is found in cranberries, blueberries, wheat and pears [53, 64, 65]. Arbutin is used as an effective treatment of hyperpigmentary disorders and displays less melanocyte cytotoxicity than hydroquinone. As for hydroquinone, arbutin inhibits melanogenesis by competitively and reversibly binding tyrosinase without influencing the mRNA transcription of tyrosinase [66]. The milder effect of arbutin compared to its mother compound, hydroquinone could be attributed to the glycoside form where the glycosidic bond needs to be cleaved prior affecting tyrosinase [67].

The synthetically produced derivate of arbutin, deoxyarbutin, has been shown to be effective and safer skin-lightening agent [59, 64, 68, 69]. Hu *et al.*, compared the effect of hydroquinone, arbutin and deoxyarbutin and found that all three compounds had similar inhibitory effects on tyrosinase activity. The protein expression of tyrosinase was not affected by arbutin nor hydroquinone, whereas an effect on the protein level was seen by deoxyarbutin. Also, less melanocyte cytotoxicity was seen by deoxyarbutin compared to the two other quinones [68]. In a human clinical trial, topical treatment with deoxyarbutin for 12 weeks resulted in a significant reduction in overall skin lightness and improvement in solar lentigines in a population of light-skinned or dark-skinned individuals, respectively [70].

Interestingly, a mechanism of *in vivo* control of quinone-mediated stress was proposed by Schallreuter *et al.* The authors found that the antioxidant system thioredoxin/thioredoxin reductase isoenzyme I/II and tetrahydrobiopterin are capable of electrochemically reducing quinones within the epidermis protecting the skin from topical applications containing quinones [71]. However, taking into consideration that fair skin individuals have low thioredoxin reductase/thioredoxin activities [72] together with low epidermal tetrahydrobiopterin levels [10], it was proposed that these individuals are more sensitive to topical applications of quinones, and therefore melanocyte toxicity could be more pronounced in this group [71].

Skin-lightening actives originating from microorganisms

There are also other non-quinone-related agents with tyrosinase-inhibiting activities such as Kojic acid (5-hydroxy-2-hydroxy-methyl-4H-pyran-4-one). Kojic acid is a naturally occurring hydrophilic fungal metabolite obtained from species of *Acetobacter*, *Aspergillus* and *Penicillium* [73]. The activity of kojic acid is believed to arise from chelating copper atoms in the active site of tyrosinase

as well as suppressing the tautomerization of dopachrome to 5,6-dihydroxyindole-2-carboxylic acid [59, 74]. Although kojic acid is a popular treatment for melasma, it can cause contact dermatitis, sensitization and erythema [65].

Azelaic acid (1,7-heptanedicarboxylic acid) is a saturated dicarboxylic acid found naturally in wheat, rye, and barley. It is a natural substance that is produced by *Pityrosporum ovale*, a yeast strain [75, 76]. It is used as a treatment for acne, rosacea, skin pigmentation, freckles, nevi and senile lentigines [57, 64, 69]. The compound is able to bind amino and carboxyl groups and may prevent the interaction of tyrosine in the active site of tyrosinase and thus function as a competitive inhibitor. Interestingly, azelaic acid has been shown to inhibit thioredoxin reductase in guinea pig and human skin, on cultures of human keratinocytes, melanocytes, melanoma cells, murine melanoma cells and on purified enzymes from *Escherichia coli*, rat liver, and human melanoma [72]. This might explain the antiproliferative and cytotoxic effect of azelaic acid as thioredoxin reductase, the synthesis of deoxyribonucleotides, the substrate for DNA synthesis in the S-phase of the cell cycle [77].

Flavonoid-like agents

There are approximately 4000 flavonoids identified to date, and this class of plant polyphenols can be found in leaves, bark and flowers. They are reported to have a variety of effects such as anti-inflammatory, antiviral, antioxidant and anticarcinogenic properties [59, 60, 75]. The main action behind the pigment-reducing effect of flavonoids may be the ROS-scavenging properties and the ability to chelate metals at the active site of metalloenzymes [60].

A number of flavonoids are frequently used in skin-lightening preparations such as aloesin, hydroxystilbene derivatives and licorice extracts.

Aloesin has been proven to competitively inhibit tyrosinase but also been shown to inhibit TH and DOPA oxidase activities [78].

Some of the more efficient pigment-lightening flavonoid subcategories are the hydroxystilbene compounds, of which resveratrol is one common example. Resveratrol is found in red wine and has been shown to reduce not only tyrosinase activity but also MITF expression in B16 mouse melanoma cells [60, 79].

Another flavonoid is licorice, more specifically glabridin, the main ingredient of the hydrophobic fraction of licorice extract. This ingredient has been shown to inhibit tyrosinase activity in B16 murine melanoma cells [80, 81].

There are some controversies however regarding the use of flavonoids in skin-lightening preparations as some flavonoids are known to increase melanogenesis. A good example of this contradiction is the citrus flavonoid naringenin, which has been shown to increase melanogenesis and the expression of melanogenic enzymes [82]. An additional described example is quercetin that was shown to induce melanogenesis in a reconstituted three-dimensional human epidermal model, where both melanin content and tyrosinase expression were markedly increased [83]. Other opposing examples of flavonoids are taxifolin and luteolin that were shown to effectively inhibit tyrosinase-catalysed oxidation of L-dihydroxyphenylalanine in cell-free extracts and in living cells and thereby reducing melanogenesis. In contrast, they attributed a stimulatory effect on tyrosinase protein levels, although the overall pigmentation was decreased [84]. Further research is needed to investigate the reason for paradoxical results for flavonoids.

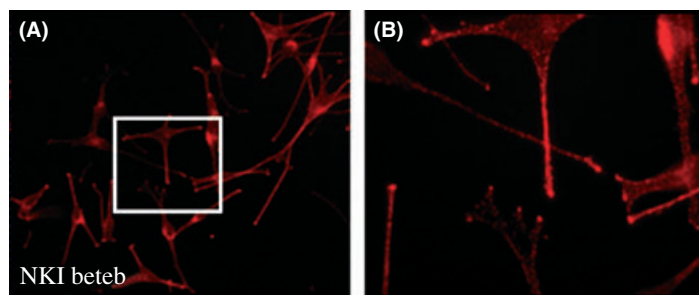


Figure 4 Human epidermal melanocytes stained for a melanosomal marker, NKI-beteb (A). Note the strong melanosomal staining in the dendritic tips at a higher magnification (B). The transfer of these melanosomes can be inhibited by specific skin-lightening ingredients.

Inhibition of melanosomal transfer

A critical component of skin pigmentation is the transfer of mature melanosomes into the keratinocytes. Transfer of melanosomes is mediated via the melanocyte dendrites to surrounding keratinocytes (Fig. 4). Even though much is known about the keratinocytes and melanocytes individually, the interactions between these cells still need to be mechanistically clarified to fully understand the transfer of melanin.

For the skin-lightening industry, melanosomal transfer has been excessively studied, and the search for actives inhibiting this action is a continuous process.

Protease-activated receptor 2 (PAR-2) inhibitors

The transmembrane G-protein-coupled receptor protease-activated receptor 2 (PAR-2) has been suggested to have an impact on melanosomal transfer. It was shown that activation of PAR-2 increased pigmentation, whereas inhibition of this receptor resulted in decreased pigmentation [85]. Within the epidermis, PAR-2 is expressed on keratinocytes only, and therefore it is believed that PAR-2 activates phagocytic capacity of keratinocytes and in this way promote melanosomal uptake [86] via cAMP and activation of the G-protein, Rho [87]. Soymilk and soybean extracts are natural skin-lightening remedies that are suggested to inhibit PAR-2 activation in the skin and result in skin lightening [88, 89].

Niacinamide

Niacinamide is a biologically active form of niacin (vitamin B3) and is found in yeast and root vegetables [90] and is an important precursor of NADH (nicotinamide adenine dinucleotide) and NADPH (nicotinamide adenine dinucleotide phosphate). These co-enzymes are found in all living cells, and the effect of niacinamide is therefore rather extensive. Several benefits in terms of improved barrier function, reduced sebum production and improved appearance of photo-aged skin including hyperpigmentation, redness and wrinkles have been described by topical usage of niacinamide [91–93].

The effect of niacinamide on hyperpigmentation is believed to occur through inhibition of melanosomal transfer. Hakozaki *et al.* showed that niacinamide has no effect on the catalytic activity of mushroom tyrosinase or on melanogenesis in monocultures of melanocytes. However, it gave 35–68% inhibition of melanosome transfer in the coculture model and reduced cutaneous pigmentation [91].

Also, lectins and their glycoconjugates have been shown to interrupt melanosome transfer. Experiments using fluorochrome

labelled melanosomes quantified by flow cytometry showed 15–44% inhibition of transfer when stimulated with lectins and neoglycoproteins [94]. The exact mechanism by which lectins are acting on melanosomal transfer remains to be elucidated.

Acceleration of epidermal turnover and desquamation

Chemical agents used to exfoliate skin are also often used as skin-lightening ingredients because they remove the uppermost layer of keratinocytes containing melanin [57]. Common examples of such agents are acids such as α -hydroxyacids, salicylic acid, linoleic acid and retinoic acids. Except for their activity on acceleration of epidermal turnover [59, 60, 65, 95–97], several of these acids have also been shown to have effect on tyrosinase. For example, α -hydroxyacids is complementing its action on desquamation with direct inhibition of tyrosinase without influencing mRNA or protein expression [65, 97, 98]. The smallest α -hydroxy acid is glycolic acid (hydroxyacetic acid or 2-hydroxyethanoic acid). Glycolic acid can be isolated from natural sources, such as sugarcane, sugar beets, pineapple, cantaloupe and unripe grapes.

Also, unsaturated fatty acids such as linoleic acid show effect on tyrosinase activity, meanwhile retinoid acids is thought to have an inhibitory effect on tyrosinase transcription [99, 100].

Another unsaturated fatty acid that demonstrates skin-lightening effects is octadecenedioic acid, which has been shown to exert its effect by binding to the peroxisome proliferator-activated receptor- γ (PPAR- γ) and thereby inhibiting the mRNA and protein levels of tyrosinase [101]. Moreover, synergistic effect of octadecenoic acid and plant extract of *Rumex Occidentalis* has been reported in reconstructed tanned epidermis [102], where the *Rumex* extract showed direct inhibition of tyrosinase activity [103].

In contrast to unsaturated fatty acids, saturated fatty acids such as palmitic acid and stearic acid have opposite action on melanogenesis, resulting in a controversial increased activity of tyrosinase together with increased melanin production [96].

Antioxidants

The idea behind using antioxidants for skin-lightening activities lies in the hypothesis that the oxidative effect of UV-irradiation contributes to activation of melanogenesis. UV irradiation can produce reactive oxygen species (ROS) in the skin that may induce melanogenesis by activating tyrosinase as the enzyme prefers superoxide anion radical (O_2^-) over O_2 [104]. Redox agents can also influence skin pigmentation by interacting with copper at the active site of tyrosinase or with o-quinones to impede the oxidative polymerization of melanin intermediates [57, 105]. Antioxidants can also

reduce the direct photooxidation of pre-existing melanin. **Common antioxidants used in skin-lightening formulations are vitamin E, vitamin C and vitamin B [106].**

Conjugates to improve the stability and effect of skin-lightening agents

Many skin-lightening actives have problems with cytotoxicity or instability during storage. Therefore, several attempts have successfully been made to synthesize conjugates to improve their properties. For example, Kojic acid has been conjugated by converting the C-7 hydroxyl group into an ester, hydroxyphenyl ether, glycoside, amino acid derivate or tripeptide derivatives [107]. In addition to increased stability, the effect can also be modified. One example of successful conjugation is kojic acid–amino acid amides leading to superior effect with 90% increased tyrosinase inhibitory activity [107]. Another derivate commonly used is magnesium ascorbyl phosphate, which is a stable derivate of ascorbic acid (vitamin C), leading to reduced skin pigmentation [108].

In addition, 3-aminopropyl dihydrogen phosphate (3-APPA) is a molecule that has been conjugated with both ascorbic acid and kojic acid. The formed molecules ascorbyl-3-aminopropyl phosphate

and kojyl-3-aminopropyl phosphate have proven to both be more stable than the individual molecules and deliver better into the skin resulting in a more efficient lightening effect [109].

Table I shows the *in vivo/in vitro* studies conducted for each skin-lightening actives.

Hypothesis for new skin-lightening targets

To date, many skin-lightening actives are tested through their tyrosinase-inhibiting effect. However, there are many pathways that could be utilized in skin-lightening attempts. In the following paragraphs, some of them are described. These mechanisms may be of interest in the search for new skin-lightening actives.

Regulation of β_2 -adrenoreceptors and catecholamines to target skin pigmentation

The classical cAMP-mediated pigmentation is thought to occur through ligand binding of POMC peptides to MC1R to increase the level of intracellular cAMP [32, 39, 42, 110, 111].

However, as studies in POMC-deficient mice have shown that these mice have black fur although they lack α -MSH and other

Table I An overview of *in vitro* and *in vivo* studies carried out for each reviewed skin-lightening ingredient

Skin-Lightening Ingredient	<i>In Vitro</i> studies	<i>In Vivo</i> studies
Hydroquinone	Inhibition of tyrosinase activity and melanin inhibition [128] and inhibition of cellular metabolism by affecting both DNA and RNA syntheses [61].	Clinical studies in patients with melasma shows reduction of pigmentation [54, 55].
Arbutin/deoxyarbutin	Inhibition of tyrosinase activity and melanin production [129], [66]. Inhibition of tyrosine hydroxylase and DOPAoxidase activities [130].	Clinical trial showed overall skin lightness and improvement in solar lentigines after 12 -week treatment [70].
Kojic Acid/Kojic acid tripeptides	Inhibition of catecholase activity of tyrosinase [74]. Comparative studies with kojic acid-tripeptides and unconjugated kojic acid [75].	Comparative study on skin-lightening effect of hydroquinone and kojic acid showed similar effects [131].
Azelaic acid	Melanin inhibition in melanoma cells [132].	Clinical study on patients with facial hyperpigmentation showed an improvement in pigment intensity by one or more grades [133]. Comparative study showed 20% azelaic acid is more effective than 2% hydroquinone in patients with melasma [128]
Aloesin	Inhibition of tyrosinase, tyrosine hydroxylase and DOPA oxidase activities. Also synergistic action with arbutin shown [78, 134].	Clinical study showed suppressed pigmentation by 34% in volar forearm [135].
Resveratrol	Reduction in MITF and tyrosinase promoter activities (transfection studies in melanoma cells) [79, 136].	No trials in humans. Dark-skinned Yucatan swine treated with resveratrol showed visible skin lightening, which was confirmed histologically [79]
Glabridin (Liquirice)	Glabrene and isoliquiritigenin in the licorice extract can inhibit both mono- and diphenolase tyrosinase activities [81]	Trial for melasma treatment using liquiritin cream showed good to excellent results in 90% of the patients [137]
Soyabean	Soyabean inhibits protease-activated receptor 2 cleavage, affects cytoskeletal and cell surface organization, and reduces keratino cyte phagocytosis [88]	Study on facial photodamage showed that soyabean is more efficient than the vehicle in improving mottled pigmentation [89]
Niacinamide	No catalytic activity of mushroom tyrosinase or on melanogenesis in monocultures of melanocytes. 35–68% inhibition of melanosome transfer in the coculture model [91]	Clinical study showed significant improvements versus control in end points: fine lines/wrinkles, hyperpigmentation spots, texture, and red blotchiness [92]
α -Hydroxyacid	Glycolic acid and lactic acid inhibited melanin formation in human melanoma cells. Tyrosinase activity was inhibited. No effect on tyrosinase, TRP-1 and TRP-2 mRNA [98]	Study on topical application of a 10% glycolic acid for melasma showed improvement in 91% of patients [138]. Comparative study with hydroquinone showed that glycolic acid is not more efficient than hydroquinone [139]
Retinoic acid	Inhibition of tyrosinase/TRP-1 protein expression concomitant with melanin synthesis [99]	Study on overall skin lightening in face showed lighter pigmentation in 68% of the patients [140]. Clinical trial on melasma showed only marginal significant pigment reduction compared to vehicle [141]
Vitamin C (magnesium ascorbyl phosphate)	Suppression of melanin formation on purified tyrosinase and in melanocytes [108]	Clinical study using magnesium-L-ascorbyl-2-phosphate cream resulted in lightening effect in 19 of 34 patients with chloasma or senile freckles [108]
octadecenedioic acid	Reduction in tyrosinase mRNA and protein expression concomitant with inhibition of melanogenesis [101]	Studies on octadecenedioic acid resulted in a more even skin tone and overall lighter skin colour [102, 142]

POMC-related peptides [112], it is likely that alternative pathways can activate intracellular cAMP and induce melanogenesis.

One alternative cAMP-dependent pathway that has been proposed to be active and turn on melanogenesis in these mice is the adrenergic receptor, especially since the POMC-deficient mice was shown to have an abnormally large adrenal gland [112, 113].

Moreover, human epidermal melanocytes express β_2 -adrenergic receptors (β_2 -AR) [51, 114], and its activation was shown to increase melanin synthesis [51, 115]. Interestingly, UV-induced melanogenesis was found to be blocked by β_2 -AR antagonists [115]. The importance of the adrenergic system in pigmentation has also been clinically shown in vitiligo where beta-adrenergic antagonist may increase the depigmentation process in this skin disorder [116].

Because of these new findings, it would be of interest to investigate whether β_2 -AR antagonists could have skin-lightening activity *in vivo*. It is also noteworthy that blockade of these receptors significantly improves wound healing [116, 117], which could have implications targeting the ageing process.

New ways to inhibit MITF

As MITF is the transcriptional regulator of tyrosinase, it obviously plays a critical role in the regulation of melanogenesis. Interestingly, glutaminergic receptors have been shown to specifically affect MITF expression dramatically. Blockage of the ionotropic glutaminergic receptors resulted in a sharp reduction in the protein expression of MITF [118]. Moreover, inhibition of these receptors caused a rapid change in melanocyte morphology with reversible retraction of melanocyte dendrites, which was associated with

disorganisation of actin and tubulin microfilaments [116, 118]. The importance of the glutaminergic system in pigment cells was also demonstrated recently where over-expression of metabotropic glutaminergic receptor 1 in mouse melanocytes led to melanocyte hyperproliferation [119]. In the light of these results, glutaminergic receptors may be a successful target for skin-lightening ingredients.

Control of pigmentation by 6(R)-L-erythro-5,6,7,8-tetrahydrobipterin (6BH₄)

6BH₄ is a rate-limiting cofactor for PAH and TH and an allosteric inhibitor for tyrosinase and hence of great importance for melanogenesis [116, 117, 120]. The activities of PAH, THI and tyrosinase are controlled by the cofactor 6BH₄ which in turn acts as the essential electron donor for PAH to produce L-tyrosine from L-phenylalanine and for THI to convert l-tyrosine to L-DOPA [36, 37]. Moreover, 6BH₄ is an allosteric inhibitor of tyrosinase [29, 38]. In support for the above-suggested 'three-enzyme theory' of melanogenesis, it has been documented that both melanocytes and keratinocytes hold the capacity for autocrine *de novo* synthesis/regulation and recycling of 6BH₄ [17].

Moreover, it was demonstrated that melanosomes contain indeed 6BH₄ as well as its isomer 7BH₄ at physiological concentrations [18, 39]. Epidermal levels of 6BH₄ correlate with skin phototypes I–VI with increasing levels from fair to the dark skin, and 6BH₄ *de novo* synthesis increases after UV exposure, supporting their close relationship with skin pigmentation [34]. In conclusion, 6BH₄ is one of the major players in the regulation of constitutive and *de novo* skin colour. More recently, 6BH₄ analogues such as 6,7-(R,S)-dimethyl-tetrahydropterine and 6-(R,S)-tetrahydromonap-

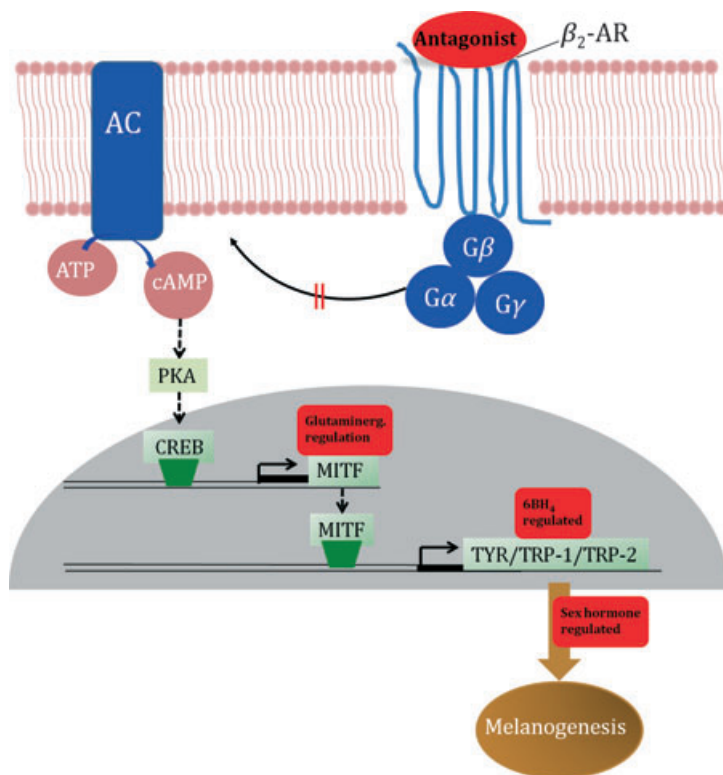


Figure 5 Illustration of four new potential transduction stages to reduce overall skin pigmentation, targeting the β_2 -adrenergic receptor using antagonists, MITF through glutaminergic regulation, regulation of 6BH₄ and control of sex hormones.

terine have been studied as possible tyrosinase inhibitors, and it has been suggested that these compounds, like 6BH₄, can act through an uncompetitive allosteric mechanism [116, 117, 121].

It has also been demonstrated that 6BH₄ (and their analogues) reduces *o*-dopaquinone non-enzymatically [116, 117, 122]. The importance of 6BH₄ in melanogenesis has also been confirmed clinically in the depigmentation disorder vitiligo, where there is an excess of 6BH₄ together with its oxidation product 6-biopterin. Because of these findings, it would be of interest to study the regulation of skin pigmentation by 6BH₄, and it is analogues to find the next generation of skin-lightening actives.

Regulation of sex hormones

Androgens affect several functions of human skin, such as sebaceous gland growth and differentiation, hair growth, epidermal barrier homeostasis and wound healing. On the other hand, oestrogens have been implicated in skin aging, pigmentation, hair growth, sebum production and skin cancer (reviewed in [123]).

A number of studies have shown that epidermal melanocytes are oestrogen responsive. However, there are conflicting reports in the literature concerning the effect of oestrogen on pigmentation.

In female guinea pigs, after ovariectomy, the melanin content of epidermal melanocytes decreases; many become smaller in size and exhibit shortened dendritic processes [124], which implicates that oestrogen would stimulate pigmentation. Furthermore, ovariectomized animals that were treated with estradiol either locally or systemically showed an increase in melanin both inside and outside the melanocytes in all regions examined [124].

In contrast, a study of male Syrian hamsters demonstrated that estradiol produced a dose-related decrease in the number of scrotal skin melanocytes [125].

Furthermore, in the same study, induced pigmentation of the costovertebral spot hair follicles by 5 α -dihydrotestosterone was reversed by estradiol.

However, after ovariectomy, the skin of female rhesus monkeys is paler, and pregnant women and women on hormonal contraception have increased prevalence of melasma [126].

Even though more work is needed to rule out the effect of sex hormones on pigmentation, it is clear that these hormones affect our skin color.

In fact, the androgen precursor hormone dehydroepiandrosterone was shown to reduce skin pigmentation by 10% in women taking this hormone orally [127]. This implicates the importance of understanding the effect of sex hormones on melanin production and that this field might be of further interest in the future.

Conclusion

Great advances have been made to understand pigment biology and the processes underlying skin pigmentation in the last decade. This research has led to development of safer and more effective skin-lightening ingredients with many still directly targeting the rate-limiting enzyme of melanogenesis, tyrosinase.

In this article, some ideas for other potential mechanistic targets for control of human pigmentation have been proposed such as control of glutaminergic/adrenergic signalling, sex hormones and regulation of tetrahydrobiopterin (Fig. 5). It remains to be investigated whether regulation of these pathways could evolve in potent and safe skin-lightening regimes for future use.

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