Di-acetyl-nor-aporphines: Novel molecules and a novel mechanism to inhibit melanogenesis

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Summary: Nor-aporphine derivatives have been discovered which interfere with the flux of Calcium into and out of the cell interior. It has been shown that adrenergic antagonists that block the Calcium exchange lead to an inhibition of Protein kinase C activity, thus blocking tyrosinase activation.

Di-acetyl-dimethoxy-methyl-nor-aporphine is a semi-synthetic molecule of natural origin with very high potency. On B16 melanocytes as well as in normal human melanocytes the decrease in melanin synthesis reaches ~50% at a level of 40 ppm in the culture medium. On a molar concentration basis, this is 50 to 70 times stronger than Kojic acid inhibition. Yet, the cell viability is not affected. Reversibility studies show that after washing out of the active compound, melanogenesis returns to normal levels. Possible mechanisms of the activity are discussed.

Tests carried out on SkinEthic® three-dimensional models of the epidermis and *in vivo* clinical studies on Asian population confirm the strong inhibition of

melanogenesis. Safety evaluation of these molecules, on the other hand, demonstrates good skin tolerance and absence of toxicity.

1. Introduction

The Asian market of cosmetic and skin care products is dominated by a strong demand for active ingredients that either lighten the complexion or reduce differences in pigmentation and render the skin tone more homogeneous.

In North America, Australia and Europe the main preoccupation of women with respect to skin pigmentation consists – apart from tanning in summer – in reducing senile lentigines ("age spots"), found on the backs of the hands, arms and cleavage, areas that are very exposed, on a daily basis, to UV radiation.

In order to respond to these demands of the market, numerous products have been developed in recent years. Following the quasi-ubiquitous prohibition of hydroquinone, a very active but toxic substance, which ultimately results in definitive destruction of melanocytes, other less harmful substances have been sought.

The objective is not, in fact, to inactivate melanocytes completely. Their protective function *vis-à-vis* UV radiation must be maintained. The cosmetic aim is to slow down the melanogenesis activity of melanocytes to obtain a lighter pigmentation yielding a clearer complexion and reducing the localized hyper-pigmentation by enhanced control of pigment migration in the epidermis.

Usual methods for down-regulating skin pigmentation via topical means are based on the competitive inhibition of the enzyme tyrosinase (arbutine, hydroquinone, Kojic acid) which is implicated at several steps in melanogenesis. Few other approaches have led to significant results in treating age spots, melasma/chloasma and skin tone.

2. Recent discoveries in melanogenesis

Numerous biological responses involve Ca^{++} as an intracellular messenger. Generally speaking, the changes in intracellular Ca_i^{++} ion concentration induce cell responses and/or modulate the enzymes involved in the pathways that are activated by other extra- or intra-cellular messengers.

The variations in Ca^{++} influx modulate the release of the intracellular Ca_i^{++} stored in intracellular vesicles [1].

In the normal mechanism of UV radiation-stimulated melanogenesis, Ca^{++} plays an important role. UV irradiation of melanocytes leads to a doubling of the intracellular Ca^{++} concentration [1]. Buffey et al. [2] showed that Ca_i^{++} increases the efficacy of α-MSH binding to receptors and the activation of adenyl cyclase and pre-tyrosinase in melanosome membranes. Yamada et al. [3] showed that high Ca^{++} concentrations were necessary in order to induce melanophore aggregation. The interesting finding is the fact that blocking of α- and β-adrenergic receptors with antagonists (yohimbin, prazosin) inhibits this reaction to calcium. These findings show the close links that exist between variations in intracellular calcium levels and cell responses to α-adrenergic membrane receptor stimulation and to α-MSH.

The α-adrenergic pathway has been quite substantially documented since it impacts both types of cell that are directly interactive in melanogenesis: keratinocytes and melanocytes.

Schallreuter's recent studies [4] have shown the presence of 7000 β-adrenergic and 4000 α-adrenergic receptors expressed on the surface of each melanocyte, if the cells are in close contact with well differentiated

keratinocytes. In that exchange, the differentiated keratinocytes secrete catecholamines which in turn regulate both calcium homeostasis (the number of adrenergic receptors expressed is correlated with the calcium influx) and melanogenesis. Catecholamines thus induce an increase in inositol triphosphate (IP3) and diacylglycerol (DAG) with, in consequence, activation of PKC-dependent tyrosinase.

Thus, the number of α-adrenergic receptors expressed on a melanocyte is related to both the calcium influx into the cell [4] and the catecholamines secreted by the keratinocyte [5].

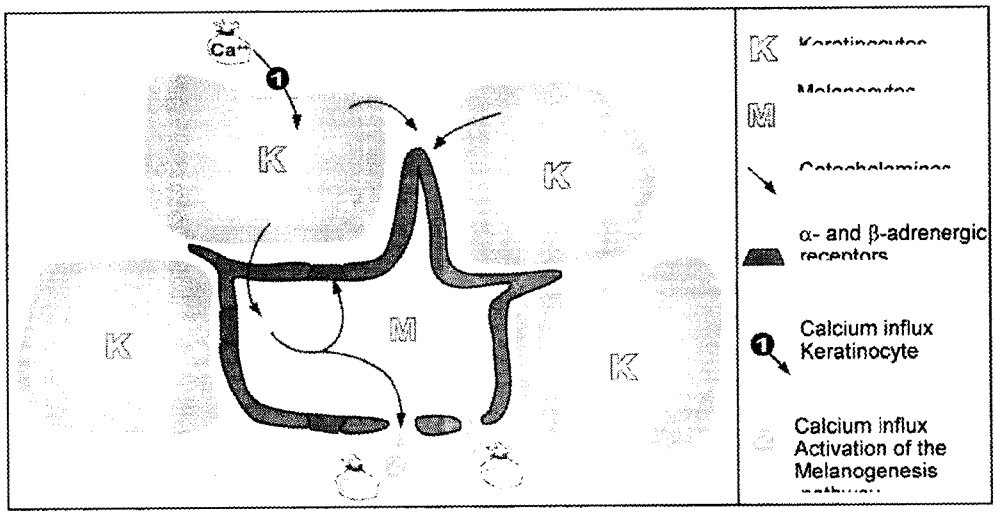

Diagram 1

The importance of the adrenergic pathway is also illustrated by Fuller's findings [6]. They showed a 90% decrease in the tyrosinase activity of human melanocytes after exposure to an α-adrenergic antagonist.

Most surprisingly, Fuller et al. also note that this inhibition is not due to substrate competition or down-regulation of enzyme biosynthesis: both the quantity of mRNA and of the enzyme remain stable in the presence of the α-adrenergic antagonist.

Consequently, the phospholipase C/IP3/PKC cascade, already addressed by Schallreuter [4] and Carsberg et al. [1] was investigated. PKC needs Ca^{++} ions in order to phosphorylate inactive tyrosinase into an active enzyme, α1-antagonists block this step via the Ca^{++} flux regulation [7]. A disturbance in calcium fluxes thus deregulates tyrosinase, which remains in the melanosome membrane in inactive pre-cursor form.

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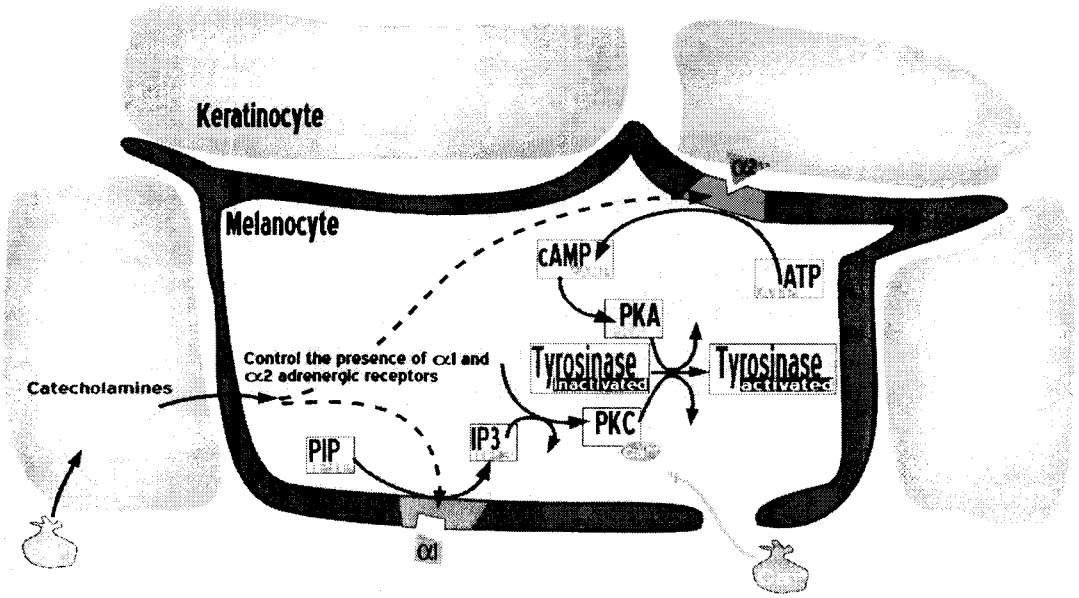
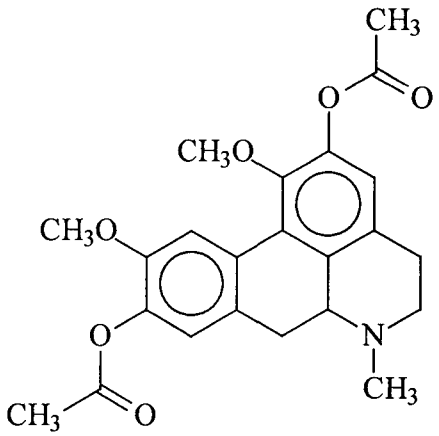


Calcium^{++} fluxes regulate keratinocyte differentiation, catecholamine release, adrenergic receptors in the melanocytes, PKC activation and thus tyrosinase activation.

Yohimbin of course cannot be used in cosmetic products; we therefore screened plant derived molecules for their potential activity to inhibit α1-adrenergic receptors and to regulate Ca^{++} flux [8-10].

Among the numerous compounds tested that had these required properties, nor-aporphines were found to be very active on a melanoma line and on human melanocytes. They also act reversibly on melanogenesis at very low concentrations. We thus investigated the melanogenesis inhibition of a particularly active derivative in more detail.



Di-acetyl-dimethoxy-methyl-nor-aporphine (DaN) [11]

3. Material and Methods

3.1. in vitro studies

3.1.1. Studies on melanocytes in culture (B16 and NHM) and reversibility

B16 cells can be used for testing and screening molecules with suspected melanogenesis inhibiting activity: the amount of melanin synthesized can be quantified and the tyrosinase enzyme activity of cell extracts can be measured. Normal human melanocytes (NHM) proliferate more slowly than transformed cell lines and, in consequence, incubation durations of 7 days are necessary. The cells were incubated in the presence of Di-acetyl-dimethoxy-methyl-nor-aporphine (DaN) or Kojic acid (KA: positive control), for 48 hours (B16) or for 7 days (NHM). The negative control incubations were conducted using culture medium alone.At time point 48h or 168 h, the cells were harvested, lysed and the protein fraction containing tyrosinase isolated by centrifuging. Tyrosinase activity was then assayed using L-dopa as substrate (dopa oxidase activity) in the absence of DaN. The dopa oxidase activity of tyrosinase was determined at 490 nm. The results of the test were then normalized by expressing the

activities per µg of protein. Parallel to this, at time point 48h or 168h, the total melanin present in the cells was determined. The cells were lysed and digested with sodium hydroxide. Colorimetric assay was conducted.

3.1.2. test on Skin-Ethi®c reconstituted skin

Photo-type VI reconstituted skin specimens were used (Skin-Ethic®, Nice, France). The skin specimens were divided into various groups and exposed to the culture medium alone or culture medium containing KA or DaN at various concentrations. After 7 days, viability was estimated by applying the MTT test (mitochondrial succinyl dehydrogenase activity) and macroscopic observation with photography conducted in order to visually assess the difference in pigmentation. Histological sections were prepared for visual evaluation of the melanin content.

3.2. *in vivo* study

The study was conducted on 20 Asian volunteers of mean age 35 years, with an equivalent phototype III, over a 2-month period from September to November 2002. Two instrumental end points were chosen: variation in the melanin index, M, by mexametry improvement in luminance, L*, and increase in ITA° angle (degree of pigmentation) by chromametry. The 20 subjects applied a cream containing 40ppm of DaN to the skin twice daily for 8 weeks.

Measurement zones: Four measurement zones were defined on the face. Two sites consisted in uniformly pigmented zones (1 and 2). If possible, the 3rd site was a hyperpigmented zone (3).The chin (4) constituted the untreated control zone. Measurements: the determinations were conducted on D0 and D56.

The results were expressed as the mean value for sites 1 and 2 for the uniform basal color of the skin; the value of site 3 for the most pigmented zone and the value for the chin as control. The mexametric result was expressed

using a 'physiological' scale extending from 80 (the lightest skins) to 500 (the darkest skins). The chromametric results were expressed on a scale from 35 (the darkest skin) to 100 (the lightest skin).

4. Results and Discussion

4.1. in vitro effects

4.1.1. Studies on melanocytes in culture (B16 and NHM) and reversibility

4.1.1.1. Effect on dopa oxidase activity

No toxicity versus control was observed for the range of concentrations tested on each molecules. The data obtained in the presence of various DaN or KA concentrations are expressed in % relative to the results obtained with the control cells. The change in dopa oxidase activity is also expressed as a percentage and the mean values were calculated for n = 4 assays.

Table I

% change (active *vs.* control) in dopa oxidase activity after incubation of cells with kojic acid (KA) or Di-acetyl-dimethoxy-methyl-nor-aporphine (DaN); n = 4; student t-test)

Concentration of active (ppm)	B16 cells exposed to KA	B16 cells exposed to DaN	Melanocytes exposed to KA	Melanocytes exposed to DaN
12	-	**-10.7 ± 19.4% ns**	-	**-18.0 ± 8.9% p<0.01**
25	-	**-44.7 ± 9.8% p<0.01**	-	**-14.0 ± 13.8% p<0.05**
37	-	**-53.7 ± 5.1% p<0.01**	-	**-22.6 ± 14.0% p<0.01**
40		-		**-35.0 ± 3.0% p<0.01**
300	**-33 ± 4.0% p<0.01**		**-46.0 ± 1.6% p<0.01**	
1000	**-60 ± 2.8% p<0.01**	-	**-67.6 ± 5.2% p<0.01**	-

Kojic acid showed dose-dependent activity resulting in ~60% inhibition. DaN was markedly more potent: in order to obtain a decrease in the standardized

quantity of dopa transformed, it was sufficient to incubate the cells with 40 ppm of the active substance, while up to 1000 ppm of Kojic acid were required to exert a similar effect.

As the activity of the enzyme was tested in the absence of DaN, the decreases in the quantity of dopa transformed cannot be due to simple competitive inhibition of the enzyme by DaN. They rather result from a decrease in the quantity of **active** tyrosinase extracted (and hence produced by the melanocytes in contact with the active substances), in agreement with the hypothesis advanced above.

4.1.1.2. Effect on melanin production

The quantities of melanin produced by and in cells exposed to DaN or KA at various concentrations were normalized for 10^6 cells, then expressed relative to the control value as a percentage.The results presented are the means for n = 4 assays.

Table II

% change in melanin content after incubation with KA or DaN at various concentrations (n=4; * n=2).

Concentration (ppm)	B16 cells exposed to KA	B16 cells exposed to DaN	Melanocytes exposed to KA	Melanocytes exposed to DaN
12	-	**-27.2 ± 17.4%** p = 0.08	-	**-28.3 ± 9.0%** p<0.05
25	-	**-60.2 ± 10.4%** p<0.01	-	**-35.0 ± 11.0%** p<0.05
37	-	**-69.3 ± 3.3%** p<0.01	-	**-40.0 ± 10.6%** p<0.01
40	-	-	-	**-51%*** p<0.01
300	**-21.7 ± 4.0%** p<0.01	-	**-37.0 ± 7.3%** p<0.01	-
1000	**-49.2 ± 2.3%** p<0.01	-	**-44.6 ± 8.2%** p<0.01	-

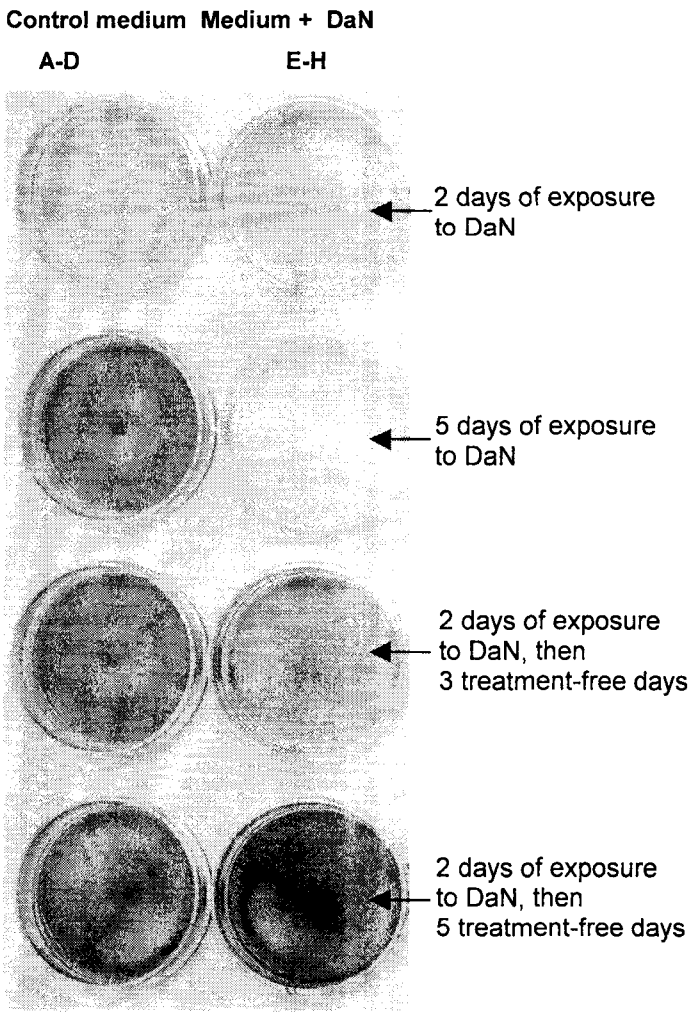
The results obtained with DaN in this protocol confirmed the previous one: the decrease in active tyrosinase is mirrored in decreased amounts of melanin synthesized. The same is true for KA.

4.1.1.3. Reversibility of the inhibitory effect on melanogenesis

To confirm that the effect of DaN on the cells was not irreversible, the following protocol was implemented: B16 cells were cultured in the presence of 20 ppm Di-acetyl-dimethoxy-methyl-nor-aporphine or in control medium. Two active substance wash-out kinetics were used: the cells were incubated with the active substance for 5 days or for only 2 days followed by a 3-day wash-out with elimination of residual DaN.

On D8, the control cultures were compared to those exposed to DaN for 2 or 5 days followed by 3 treatment-free days.

At each stage, D0, D2 and D5, the melanogenesis in the cells was visually evaluated by incubating the cells with L-dopa. The conversion into dopachrome by the tyrosinase enzyme is readily observed. Activity regularly increased for the controls up to D8 (A, B, C, D). Tyrosinase was active and continuously converted L-dopa.



For the cells exposed to 25 ppm DaN for 2 days, the tyrosinase activity was weaker (E). The reduced activity was even more marked when the cells were exposed to DaN for 5 days (F).

However, when DaN was eliminated after D2 and the medium replaced by control medium, dopa oxidase labeling began to increase again 3 days later (G), i.e. D5, reaching a level equivalent to that of the control at D8.

After a lag time, tyrosinase activity is thus fully recovered as shown by the photos. The effect of DaN on tyrosinase activity is clearly reversible.

In order to further bolster the hypothesis of a different mechanism of action, the effect of DaN on isolated tyrosinase enzyme in a cell-free medium was investigated, in comparison to the inhibitory properties of catalytic (Kojic acid) or competitive (arbutin) action. DaN shows no inhibition of the isolated tyrosinase enzyme (data not shown).

This results is similar to the observations by Fuller et al. [6]: the α-antagonist yohimbine inhibits 90% of the intracellular tyrosinase activity in melanocytes, but does not interfere with isolated human tyrosinase and its L-dopa oxidase activity.

Fuller et al. formulated the hypothesis that the α-antagonist interferes with the activation system of the enzyme present in the melanosome: the enzyme remains in an inactive form, probably because of the inability of phosphokinase C (PKC) to carry out tyrosinase phosphorylation.

4.1.2. Studies on Skin-Ethic® reconstituted skin

The reconstituted skin model with melanocytes (SKIN ETHIC®) is a recognized tissue model that has been validated as being equivalent to living epidermis. The model constitutes the best approximation of the real conditions

of melanogenesis, where regulation is mediated by the keratinocyte-melanocyte interaction. The importance of that interaction with respect to melanisation is now known [5]. The MTT viability test showed good epidermal tolerance of DaN tested at 10 and 40 ppm concentration.

4.1.2.1. Effect on melanisation after 7 days of exposure

The photographs of the epidermis cross sections (transverse sections of 5µ thickness, stained with Fontana-Masson) taken after 7 days of incubation with DaN are shown below:

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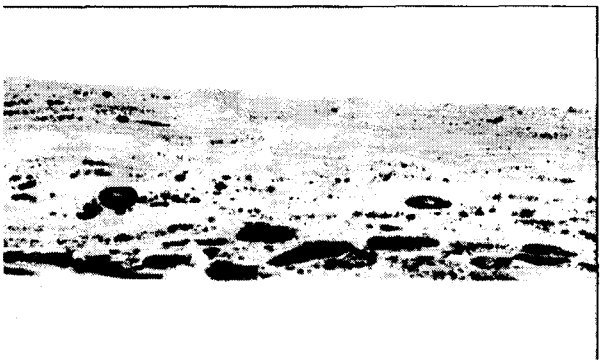
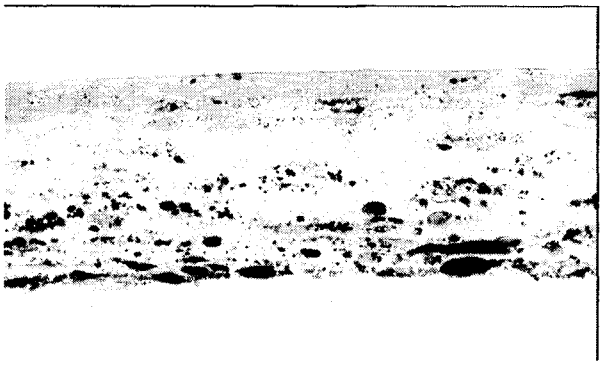
Picture A (control)



The staining of the epidermis by melanin pigments was clearly visible at basement membrane level and well distributed through the various layers of the epidermis and *stratum corneum*.

Picture B: 10 ppm of DaN



A general decrease of melanin distribution is observed.

Picture C: 40 ppm of DaN:

Exposure to DaN very markedly decreased melanin synthesis by basement membrane cells. There was also a decrease in the distribution in the supra-basal layers.

4.2. Clinical trial: Skin Lightening effect on Asian skin

4.2.1. Melanin index variation (mexametry)

The parameter M reflects the skin's melanin content by measuring the reflection of 2 wavelengths emitted by the probe and associated with the melanin-pigment absorption spectrum.

Table III

Change in melanin index (M) after 56 days of application of an emulsion containing 40 ppm DaN to the facial skin of Asian subjects. Mean results (n = 20).

M index	**Normal zone**		**Pigmented zone**		**Control zone**	
	T0	T56	T0	T56	T0	T56
Mean	189±30	172±25	222±51	202±39	220±29	214±25
Change, T0-T56	-17 units		-20 units		-6 units	
Significance/T0	high (p<<0.001)		High (p<<0.001)		NS	
Significance/ Control zone	p<0.01		p<0.01			

While the untreated zone only showed a weak, non-significant reduction between D56 and D0, a marked 17-unit reduction was observed for the normal

zone and a 20-unit reduction for the hyperpigmented zone after 56 days of treatment. This compares favorably with a similar study where 20 Asian volunteers used a 1% Kojic acid cream (i.e. 10.000ppm) for 2 months (twice daily) under similar conditions (unpublished data).

Table IV

Comparison of the *in vivo* results on melanin index, M, after 56 days of twice daily application of 10.000ppm KA or 40ppm DaN

Melanin index, M	% SUBJECTS IMPROVED	
	10.000ppm Kojic acid	40ppm Di-acetyl-dimethoxy-methyl-nor-aporphine
Normal zone	45%	
Hyperpigmented zone	45%	
Normal zone		85%
Hyperpigmented zone		90%
Significance	NS for the 2 zones	p<0.001 for the 2 zones

4.2.2. Variations in the Luminance (clarity) L* and ITA° (chromametry)

The L* and ITA° parameters were taken into account in this study.

L* expresses luminance on a black-white scale. The value increases as the skin lightens. ITA° is a complex parameter combining L* and a blue-yellow chromaticity parameter. The parameter is expressed in degrees and ranges from 10° (very dark) to >55° (very light). A lightening effect thus gives rise to an increase in the ITA° value.

The variations in the ITA° angle D0 vs. D56, are shown in the following table:

Table V

Change in ITA° after 56 days of application of an emulsion containing 40 ppm of DaN to the facial skin of Asian subjects. Mean results (n = 20).

ITA°	Normal zone		Pigmented zone		Control zone	
	T0	T56	T0	T56	T0	T56
Mean	37.2°±5.8	39.2°±3.9	30.0°±7.	32.9°±5.6	31.8°±5.6	33.0°±5.1
Variation, T0-T56	+2°		+2.9°		+1.2°	
Significance/T0	p<0.05		p<0.01		NS	

The L* values (data not shown) follow the changes in ITA° very closely. While the untreated zone did not show any significant change for luminance L* or for ITA°, a significant improvement in both criteria was observed in 65 to 80% of the volunteers after 8 weeks of application. Thus, luminance improved from +0.8 to 1.2 units for the normal and hyperpigmented zones respectively and ITA° improved, on average, by 2 to 3° in the normal and hyperpigmented zones. In certain subjects, the latter increase reached 11° in the normal zone and 8.7° in the hyperpigmented zone. Those values are close to a change in phototype classification.

5. Conclusion

Melanogenesis is an extremely complex process in the epidermis, which is regulated by many control mechanisms. Peptide hormones (α-MSH), adrenergic substances, Calcium ions, cAMP and many enzymes play key roles in skin pigmentation. Increasing knowledge and understanding of these pathways make it possible to search for new substances that can be used topically, in cosmetic products where efficacy and safety are the major concerns. A new class of molecules, nor-aporphines, has been discovered which allow us to attack the problem from a new angle: reversible inhibition of tyrosinase activation, rather than simple competitive inhibition of the active enzyme or outright cytotoxic effects on melanocytes. One representative of this class, Di-acetyl-dimethoxy-methyl-nor-aporphine, described in this paper, is a promising candidate for further research into safe and efficacious skin lightening effects.

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