Skin whitening agent targets MC-1 receptor

Asia is the largest and fastest growing market for whitening cosmetic products. For Asians, the brightening of the skin is considered the ideal of beauty and is associated with higher social status. Melanin absorbs harmful UV radiation and transforms the energy into harmless heat. This property enables melanin to release more than 99% of the absorbed UVR as heat. This photo-protection prevents the DNA damage that is responsible for malignant melanoma and various skin cancers. However, despite many advantages, people generally consider pigmentation undesirable. Therefore, there is strong need for an agent that inhibits melanin production thereby providing brighter skin tone, while preserving all other protective properties that melanin provides.

To meet this need, Miwon has developed and patented a new skin whitening agent, methyl undecenoyl leucinate (Fig. 1), an active ingredient of DermaPep UL. Methyl undecenoyl leucinate provides even pigmentation against age spots (melasma) as well as general skin-whitening activity. Methyl undecenoyl

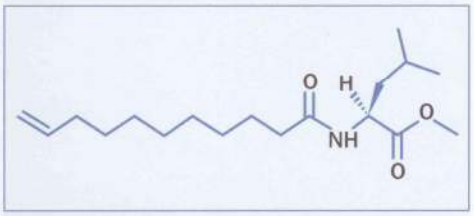


Figure 1: *Methyl undecenoyl leucinate.*

leucinate interacts with MC1R, a receptor for α-MSH, competes against its natural ligand, α-MSH, and thereby prevents any further activation of melanogenic genes such as tyrosinase, TRP1, TRP2 (DCT), MITF and POMC and finally blocks melanin synthesis.

Mechanism of whitening action

Melanin, the end-product of complex multistep transformations of L-tyrosine, is the major factor of skin and hair-darkening and provides protection from damage by UVR.

The melanocortin 1 receptor (MC1R), encodes seven-transmembrane G-protein coupled receptors, is expressed predominantly by melanocytes and is a key protein that regulates melanin synthesis. The stimulation of the MC1R is up-regulated by a-melanocyte stimulating hormone (α-MSH) and down-regulated by agouti signal protein (ASP/ASIP). α-melanocyte stimulating hormone (α-MSH) is a tridecapeptide derived from the proopiomelanocortin (POMC) by post-translational processing. This molecule serves as the source for several peptide hormones such as adrenocorticotrophin (ACTH), α-MSH, β-MSH and γ-MSH, and the endogenous opioids including β-endorphin. C- and N-terminal fragments of α-MSH have significant melanotropic effects. α-MSH/MC1R signalling mechanism is involved in the control of tyrosinase activity, melanin synthesis and melanosome transfer. α-MSH-bound MC1R activates adenylyl cyclase (AC), inducing cyclic AMP productions. This cascade involves the activation of protein kinase A (PKA) and cAMP responsive-element-binding protein (CREB) transcription factor, leading to transcriptionally activate various genes including those encoding microphthalmia associated transcription factor (MITF), the melanocyte-specific transcription factor crucial for expression of numerous melanogenic enzymes and melanocyte development/differentiation factors such as tyrosinase, TRP1, and TRP2 (DCT) which result in an elevated melanin synthesis (Fig. 2).

In vitro and *in vivo* study results showed that methyl undecenoyl leucinate is far more effective than other known whitening ingredients such as kojic acid and arbutin. In addition to its effect on melanocytes, methyl undecenoyl leucinate provided strong anti-inflammatory activity against harmful UV on human keratinocytes.

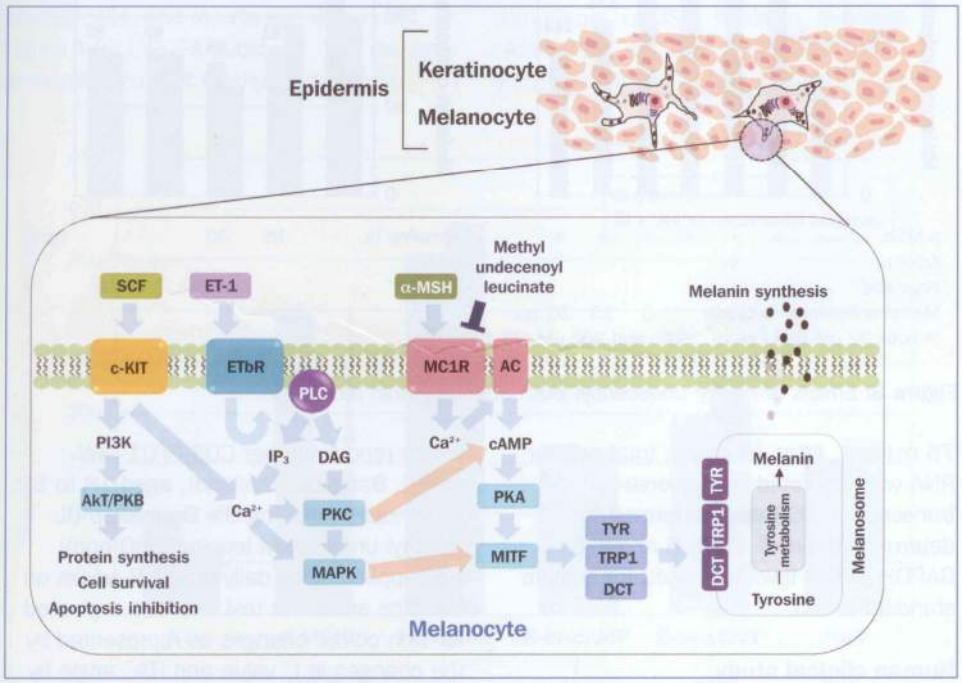


Figure 2: *General melanogenesis mechanism and inhibitory actions of methyl undecenoyl leucinate.*

Materials and methods

Melanin contents assay

Melanoma B16F10 (ATCC) or human primary melanocyte HEMn-LP (Cascade biology) cells were pre-treated with arbutin, kojic acid, or various concentrations of methyl undecenoyl leucinate for 30 minutes and then stimulated with or

without 1 ppm α-MSH. After 48 hours, the cells were washed with PBS and dissolved in 100 µL of 2 N NaOH containing 50% DMSO at 60°C and absorbance was measured at 475 nm using an ELISA reader.

Tyrosinase activity assay

Tyrosinase activity was examined in melanoma B16F10 and human primary melanocyte HEMn-LP cells. After treatment with arbutin, kojic acid or various concentrations of methyl undecenoyl leucinate, the cells were stimulated with or without 1 ppm α-MSH. After 72 hours, cells were removed from culture dishes and washed with PBS. Then, the cells were lysed with 1% Triton X-100, 1 mM PMSF, 50 mM Tris-HCl (pH7.0), 2 mM EDTA (pH8.0) and 150 mM NaCl. The lysates were centrifuged (14,000 g, 20 min, 4°C) to obtain the supernatant as a source of tyrosinase. The reaction mixture containing 50 mM phosphate buffer (pH 6.8), 0.2% L-DOPA, and the supernatant was incubated at 37°C. After incubation, dopachrome formation was assayed by measuring absorbance at 492 nm by spectrophotometer.

ELISA (Enzyme-linked immunosorbent assay)

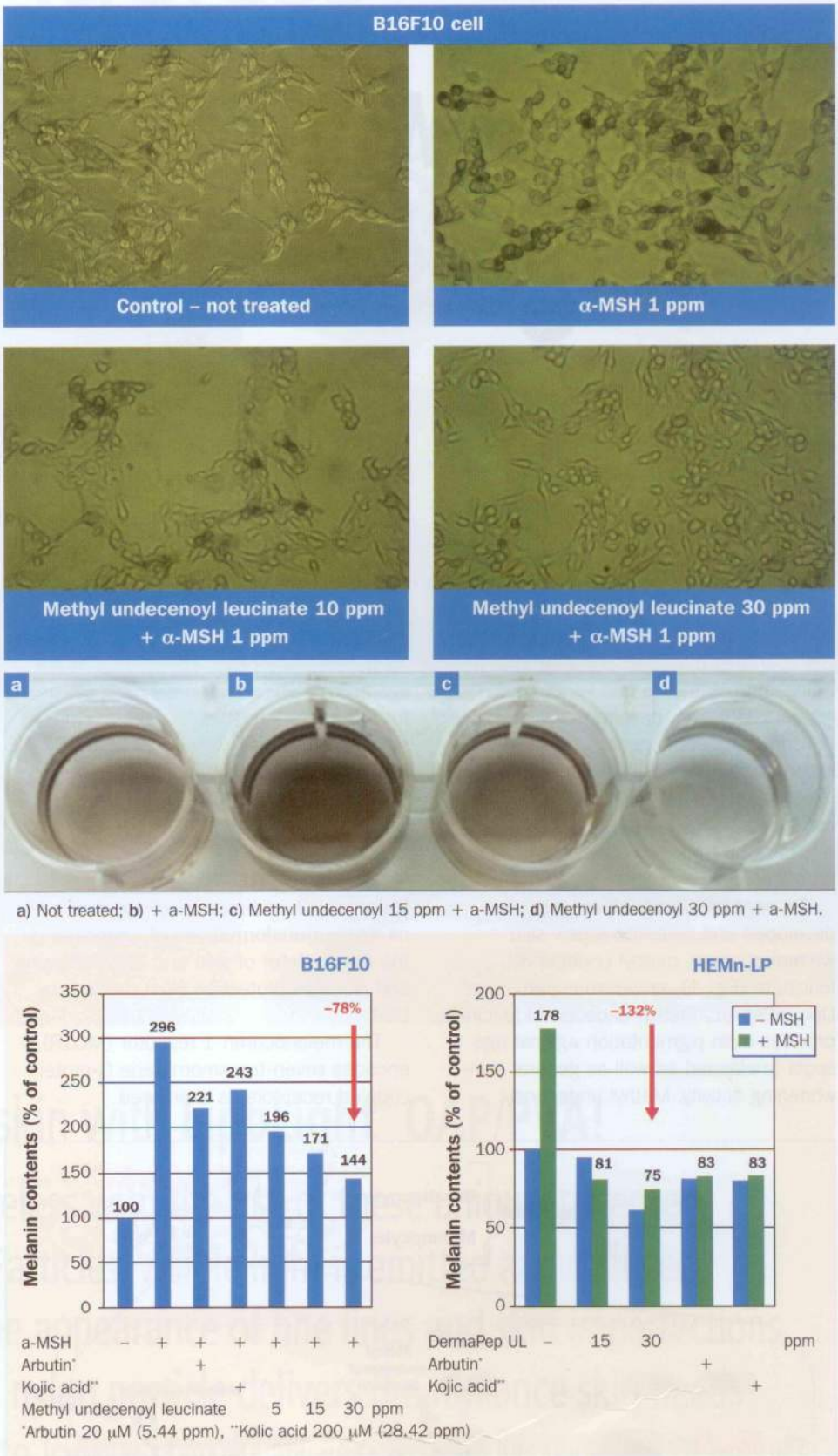
The intracellular level of cAMP was measured by direct ELISA kit (Assay Design, US). Melanoma B16F10 cells were exposed to arbutin, kojic acid or methyl undecenoyl leucinate for 20 minutes and were lysed. The intracellular cAMP was measured at 405 nm by spectrophotometer.

The production of IL-1β and IL-6 was determined by enzyme-linked immunosorbent assay (ELISA). Human keratinocyte HaCaT cells were pre-treated with methyl undecenoyl leucinate or undecylenoyl phenylalanine and then stimulated with or without UVB 75 mJ/cm². After 48 hours, cell lysates were transferred into immuno-well plates and analysed. The absorbance was measured at 450 nm by an ELISA reader.

Measurement of mRNA level (RT-PCR)

Melanoma B16F10 cells were treated with various concentrations of methyl undecenoyl leucinate and then stimulated with or without 1 ppm α-MSH. After 48 hours, total cellular RNA was extracted using Easy-spin Total RNA extraction kit (Intron) and reverse-transcription PCR was performed to determine tyrosinase, POMC, MITF, TRP1 and TRP2. β-actin mRNA level was used for sample standardisation.

Human keratinocyte HaCaT cells were treated with methyl undecenoyl leucinate and then stimulated with or without UVB 75 mJ/cm². After 48 hours, total cellular RNA was extracted and reverse-transcription PCR was performed to determine IL-1α, IL-1β, IL-6 and IL-8. GAPDH mRNA level was used for sample standardisation.



a) Not treated; b) + a-MSH; c) Methyl undecenoyl 15 ppm + a-MSH; d) Methyl undecenoyl 30 ppm + a-MSH.

Figure 3: *Effects of methyl undecenoyl leucinate in melanin production.*

Human clinical study

Clinical study was conducted on 30 Asian healthy female volunteers (Spincontrol Asia [study report number C03-PF01-MW-AL09], Bangkok, Thailand), aged 18 to 52. A formula containing 3% DermaPep UL (methyl undecenoyl leucinate 30 ppm) was applied twice daily during 8 weeks on the face area. The test site was evaluated for skin colour changes as represented by the changes in L* value and ITA° angle by chromameter (Chromameter CR-300, Minolta, Japan).

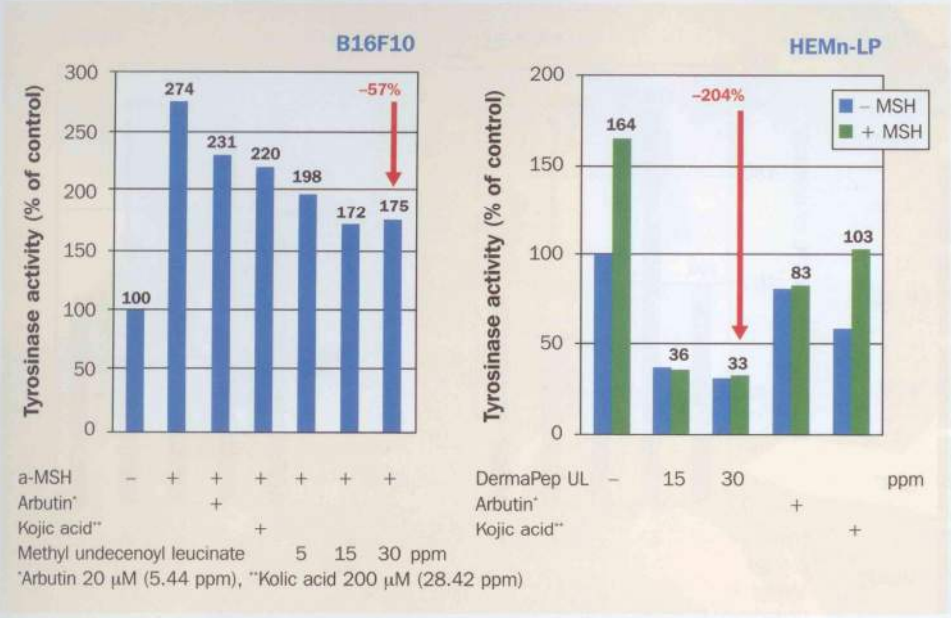


Figure 4: *Effects of methyl undecenoyl leucinate in tyrosinase activity.*

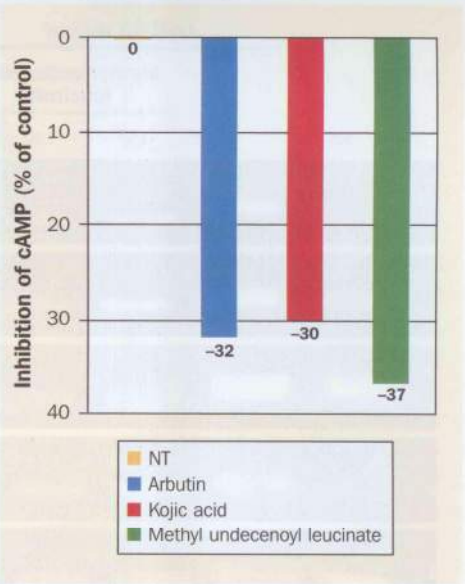


Figure 5: *Effects of methyl undecenoyl leucinate in cAMP release.*

Results

Inhibition of melanin production

Visible changes in melanin content were observed in cell cultures incubated with methyl undecenoyl leucinate (Fig. 3). The proliferation rate of cultured B16F10 melanoma cells was not altered during the 72 hours in the treatment of methyl undecenoyl leucinate. Even at 15 μg/mL use level, methyl undecenoyl leucinate strongly inhibited α-MSH-induced melanin synthesis. In addition, methyl undecenoyl leucinate showed far better depigmenting effect than other known whitening agents such as arbutin and kojic acid.

Inhibition of tyrosinase activity

Since tyrosinase is a rate-limiting enzyme in melanin synthesis, tyrosinase activity was determined in methyl undecenoyl leucinate-treated cells, and results are shown in Figure 4 for L-DOPA substrate. The results demonstrated that methyl undecenoyl leucinate has a significant inhibitory effect against tyrosinase using L-DOPA as a substrate. The efficacy of methyl undecenoyl leucinate was far better than that of the reference ingredients.

Inhibition of cAMP activation

To examine the inhibitory effect of methyl undecenoyl leucinate on cAMP activation, the intracellular level of cAMP was measured by direct ELISA. Methyl undecenoyl leucinate efficiently blocked cAMP production which is a direct down-stream target of MC1R (Fig. 5).

Methyl undecenoyl leucinate as a MC-1R specific antagonist

To confirm whether methyl undecenoyl leucinate is MC1R-specific, B16F10 were activated with several melanogenenic stimulators. α-MSH, forskolin, 8-bromo-cAMP, PMA, and 8-bromo-cGMP, specific activators for MC1R, AC, cAMP, PKC and PKG respectively. Melanin produced only through the activation by α-MSH were significantly suppressed by methyl undecenoyl leucinate (Fig. 6).

Inhibition of melanogenic gene expression

To assess methyl undecenoyl leucinate-induced modulation of melanogenic mRNA expression level, RT-PCR was performed on tyrosinase, TRP1, TRP2 (DCT), MITF and POMC (Fig. 7). Methyl undecenoyl leucinate significantly down-regulated the expression of melanogenic genes.

Anti-inflammatory effect of methyl undecenoyl leucinate

To examine the anti-inflammatory effect of methyl undecenoyl leucinate, human keratinocytes were stimulated by UVB irradiation. Methyl undecenoyl leucinate significantly inhibited UV-induced expression and production of pro-inflammatory cytokines (Figs. 8 & 9).

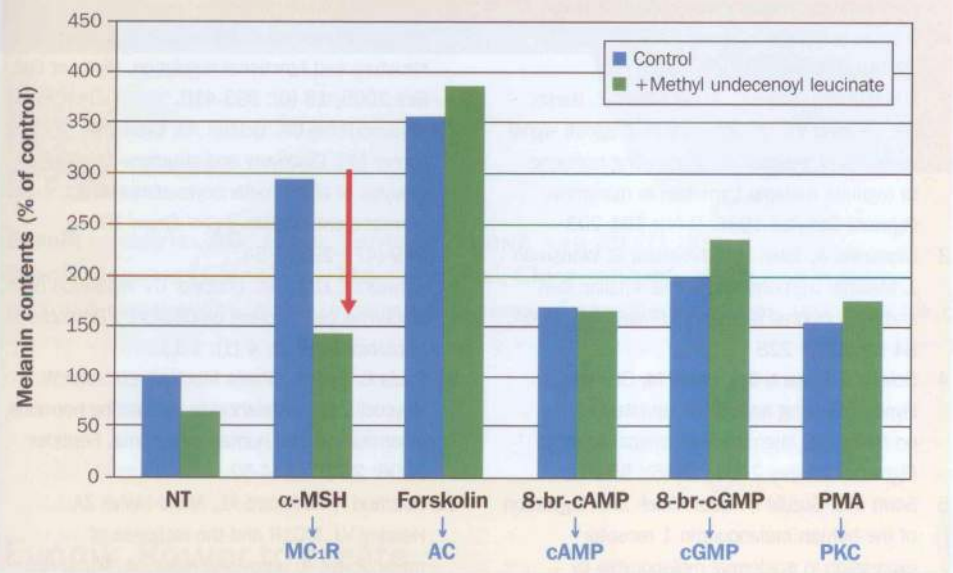


Figure 6: *Methyl undecenoyl leucinate as a MC1R-specific antagonist.*

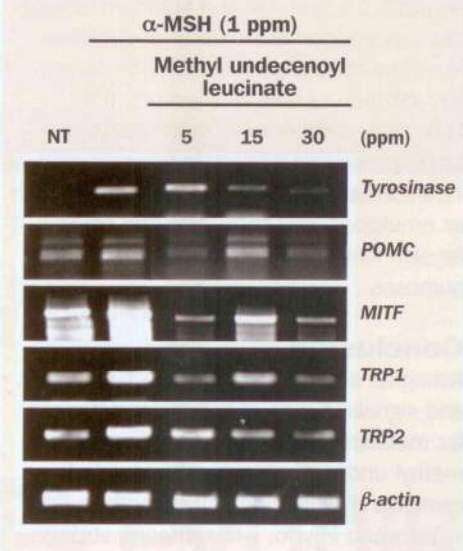


Figure 7: *Effects of methyl undecenoyl leucinate in melanogenic genes expression.*

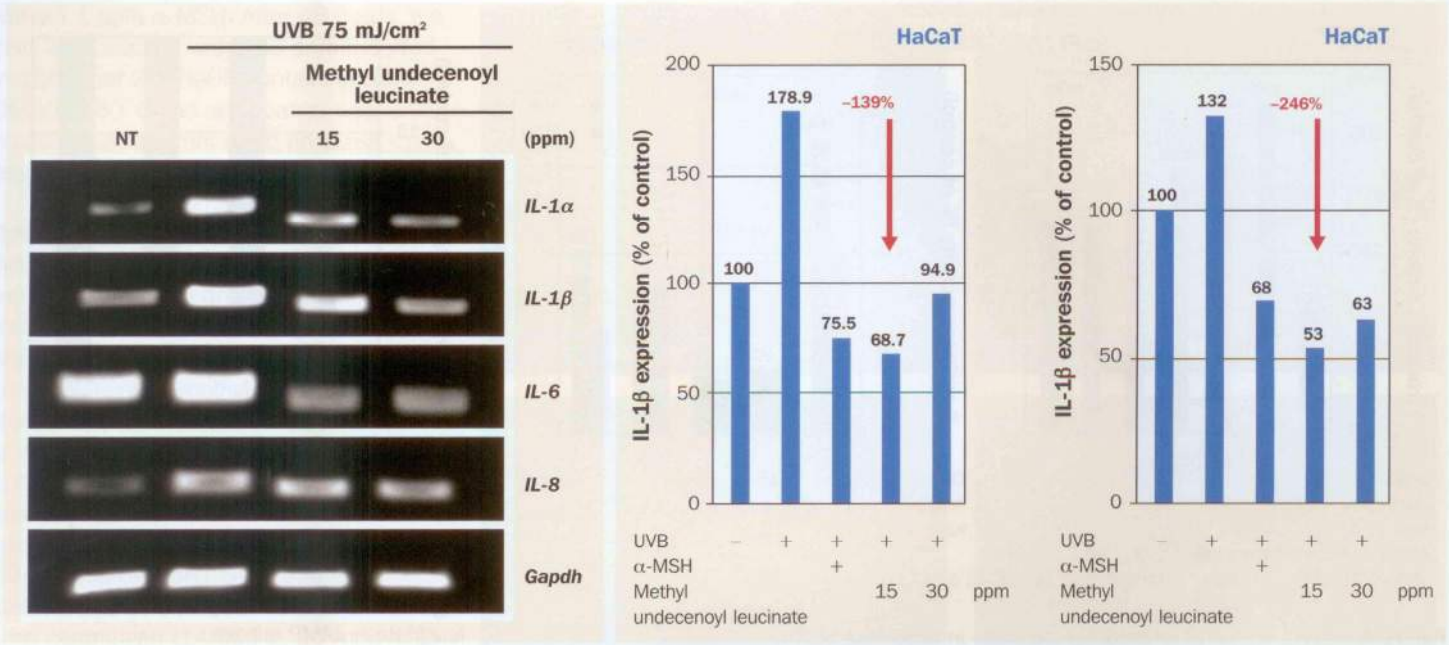


Figure 8: *Effects of methyl undecenoyl leucinate in inflammatory cytokine expression.*

Clinical study

The formula containing 3% of DermaPep UL was applied twice daily for 8 weeks on the face area of 30 Asian individuals, aged 18 to 52. The test site was evaluated for skin colour change as represented by the change in L values and ITA°.

L* = Luminosity (Lightness).

ITA° = Arc tan ((L*-50)/b*) 180π.

Increase of L* and ITA° indicates a decrease in skin pigmentation. As shown in Figure 10, after 56 days, L* value increased about 1.2 and ITA° angle about 8.6%.

Cosmetic Uses

DermaPep UL (INCI/CTFA-Declaration: Methyl Undecenoyl Leucinate, (and) Glycerin (and) Butylene Glycol) has an active ingredient content of 1% methyl undecenoyl leucinate (CAS No.1246371-29-8). Its recommended concentration is between 2% and 4% and standard testing has been performed on the product that has showed neither cytotoxic effects nor any irritation or sensitisation reaction in healthy volunteers with an occlusive single patch test. It can therefore be incorporated in cosmetic formulations such as emulsions, oily sera, gels and creams for skin whitening and anti-inflammatory purposes.

Conclusion

Acting as an antagonist of MC-1 receptor and signalling, one of the major pathways for melanin production and pigmentation, methyl undecenoyl leucinate provided exceptional whitening activity both in *in vitro* and *in vivo*, while offering strong anti-inflammatory activity against harmful UV light. PC

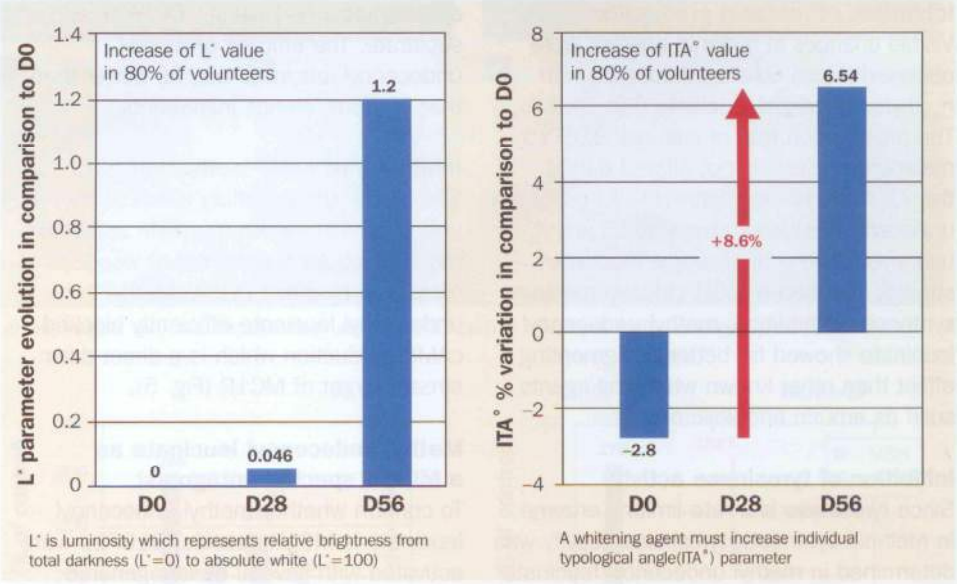


Figure 9: *Changes in L* and ITA° values* (in vivo *clinical study).*

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