Development of Natural Antioxidants and Whitening Agents for Cosmeceuticals

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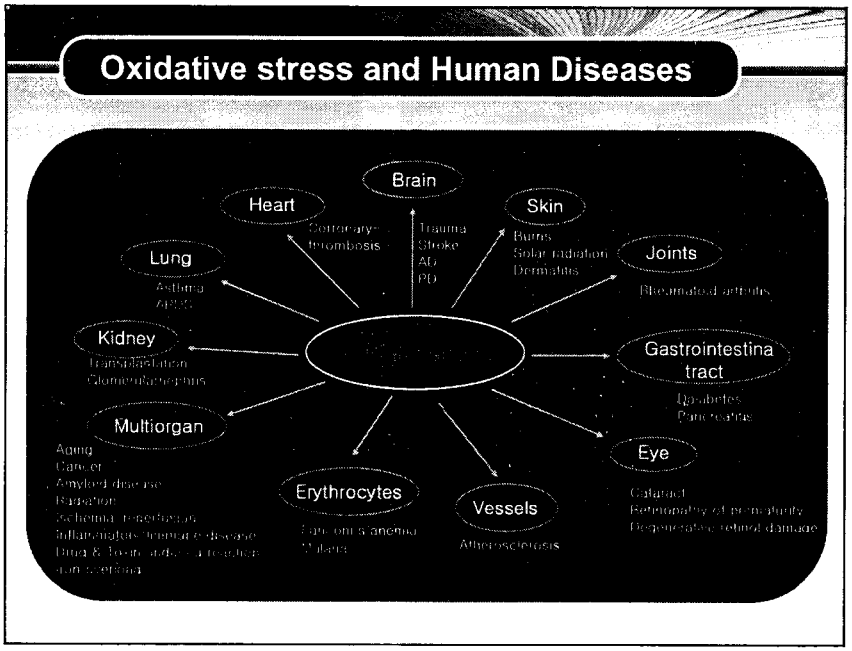
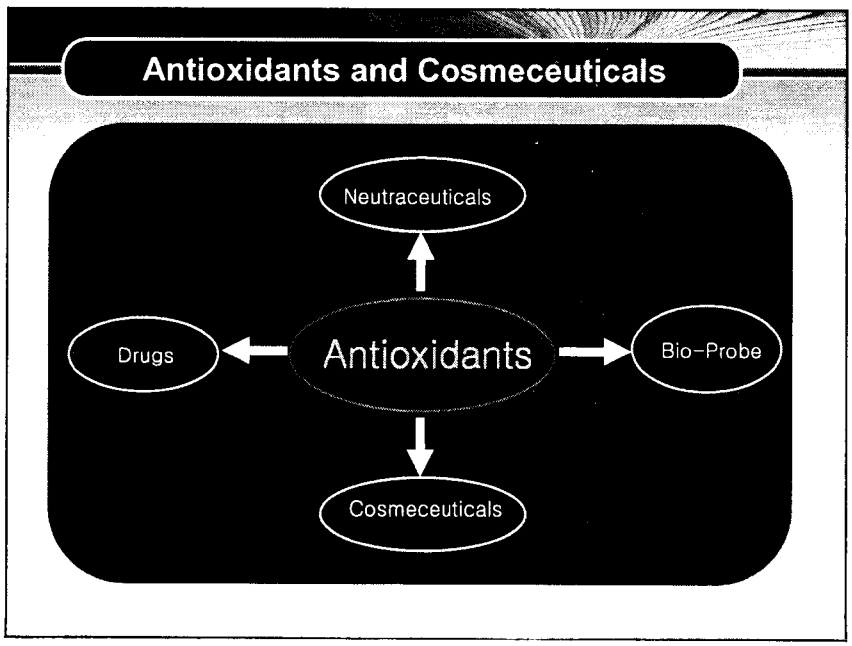
Abstract

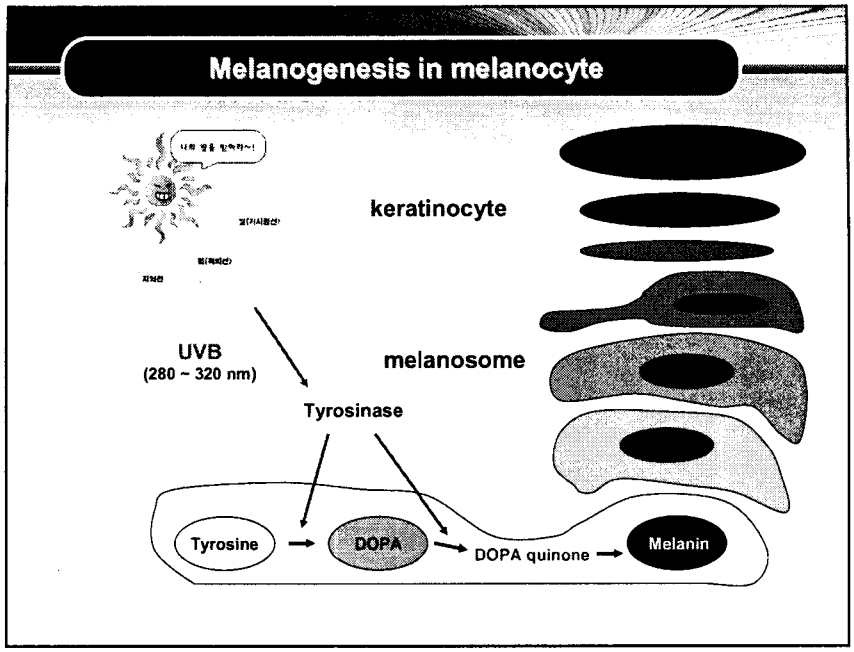
Oxidative stress have known to be a risk factor for the degenerative processes and closely related to a lot of diseases. It is well established that antioxidants are good in protection and therapeutic means against oxidative damage. There is increasing interest in natural antioxidants and many natural antioxidants have been found and utilized as the possible protection for various diseases and skin aging. We have screened natural antioxidant agents for cosmeceuticals, nutraceuticals, and drugs as therapeutic and preventive means against oxidative stress, and have developed a number of novel antioxidants from various natural sources. A novel melanin synthesis inhibitor, Melanocin A, isolated from the metabolite of a fungal strain *Eupenicillium shearii* F80695 inhibited mushroom tyrosinase and melanin biosynthesis of B16 melanoma cells with IC_{50} value of 9.0 nM and MIC value of 0.9 μM, respectively. Melanocin A also exhibited potent antioxidant activity by scavenging of DPPH and superoxide anion radicals. UV was found to increase the level of hydrogen peroxides and other reactive oxygen species (ROS) in skin tissues. This increase in ROS may not only alter the structure and function of many genes and proteins directly but may also modulate their expressions through signal transduction pathways and, ultimately, lead to skin damage. We investigated the effect of Melanocin A on UV-induced premature skin aging. Firstly, the effect of Melanocin A on UV-induced matrix metalloproteinase (MMP)-9 expression in an immortalized human keratinocyte cell line, HaCaT in vitro was investigated. Acute UV irradiation induced MMP-9 expression at both the mRNA and protein levels and Melanocin A suppressed this expression in a dose-dependent manner. We then

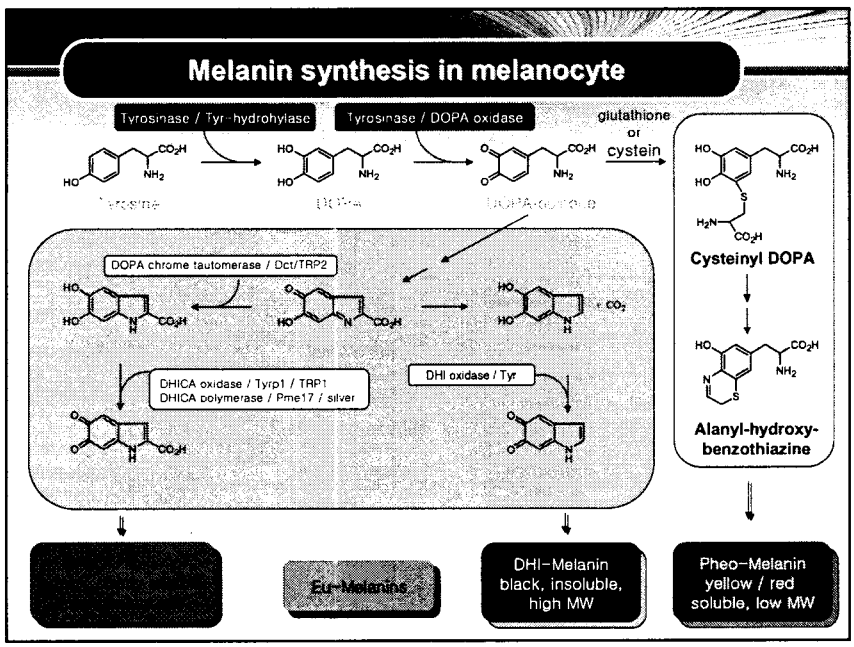
investigated UV-induced skin changes in hairless mice in vivo by Melanocin A. Chronic exposure of hairless mouse dorsal skin to UV increased skin thickness and induced wrinkle formation and the gelatinase activities of MMP-2 and MMP-9. Moreover, Melanocin A significantly suppressed UV-induced morphologic skin changes and MMP-2 and MMP-9 expression. These results show that Melanocin A can prevent the harmful effects of UV that lead to skin aging. Therefore, we suggest that Melanocin A should be viewed as a potential therapeutic agent for preventing and/or treating premature skin aging. Terrein is a bioactive fungal metabolite isolated from *Penicillium* species. Terrein has a relatively simple structure and can be easily synthesized. However, the biologic effects of terrein are comparatively unknown. We found for the first time that terrein potently inhibit melanin production in melanocytes and has a strong hypopigmentary effect in a spontaneously immortalized mouse melanocyte cell line, Mel-Ab. Treatment of Mel-Ab cells with terrein (10–100 mM) for 4 days significantly reduced melanin levels in a dose-dependent manner. In addition, terrein at the same concentration also reduced tyrosinase activity. We then investigated whether terrein influences the extracellular signal-regulated protein kinase (ERK) pathway and the expression of microphthalmia-associated transcription factor (MITF), which is required for tyrosinase expression. Terrein was found to induce sustained ERK activation and MITF down-regulation, and luciferase assays showed that terrein inhibits MITF promoter activity in a dose-dependent manner. To elucidate the correlation between ERK pathway activation and a decreased MITF transcriptional level, PD98059, a specific inhibitor of the ERK pathway, was applied before terrain treatment and found to abrogate the terrein-induced MITF attenuation. Terrein also reduced the tyrosinase protein level for at least 72 h. These results suggest that terrain reduces melanin synthesis by reducing tyrosinase production via ERK activation, and that this is followed by MITF down-regulation.

Key words: Melanocin A, Terrein, antioxidant, melanin synthesis, UV-induced skin aging


Oxidative stress and Human Diseases
Brain
Heart
Skin
Lung
Joints
Kidney
Gastrointestina tract
Multiorgan
Eye
Erythrocytes
Vessels
Trauma
Stroke
AD
PD
Burns
Dermatitis
Asthma
Diabetes
Cataract
Atherosclerosis


Antioxidants and Cosmeceuticals
Neutraceuticals
Drugs
Antioxidants
Bio-Probe
Cosmeceuticals


Melanogenesis in melanocyte
keratinocyte
melanosome
UVB
(280 ~ 320 nm)
Tyrosinase
Tyrosine
DOPA
DOPA quinone
Melanin


Melanin synthesis in melanocyte
Tyrosinase / Tyr-hydrohylase
Tyrosinase / DOPA oxidase
glutathione
or
cystein
Cysteinyl DOPA
Alanyl-hydroxy-
benzothiazine
DOPA chrome tautomerase / Dct/TRP2
DHICA oxidase / Tyrp1 / TRP1
DHICA polymerase / Pme17 / silver
DHI oxidase / Tyr
Eu-Melanins
DHI-Melanin
black, insoluble,
high MW
Pheo-Melanin
yellow / red
soluble, low MW

Purification procedure for melanocins A~ D

Culture of *Eupenicillium shearii 80695*

Broth filtrate
extracted with EtOAc
concentrated *in vacuo*

Mycellial cake
extracted with 70%aq. acetone
concentrated *in vacuo*
extracted with EtOAc

↓ Ethylacetate extrat

↓ Silica gel column chromatography
eluted with $CHCl_3$:MeOH 15:1 ~ 2:1

↓ Sephadex LH-20 column chromatography
eluted with MeOH

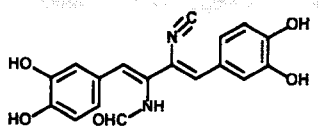
↓ ODS column chromatography
eluted with 5 ~ 100% aq. MeOH

↓ Sephadex LH-20 column chromatography
eluted with MeOH

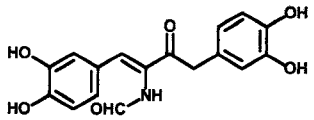
↓ HPLC (ODS)

Melanocins A, B, C, D → **Structure determination by NMR, Mass, UV and IR spectroscopy**

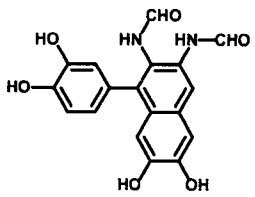
Chemical structures of melanocins A - D



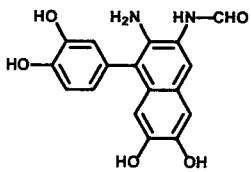
Melanocin A



Melanocin B

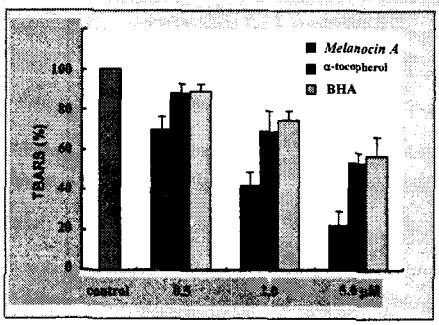


Melanocin C



Melanocin D

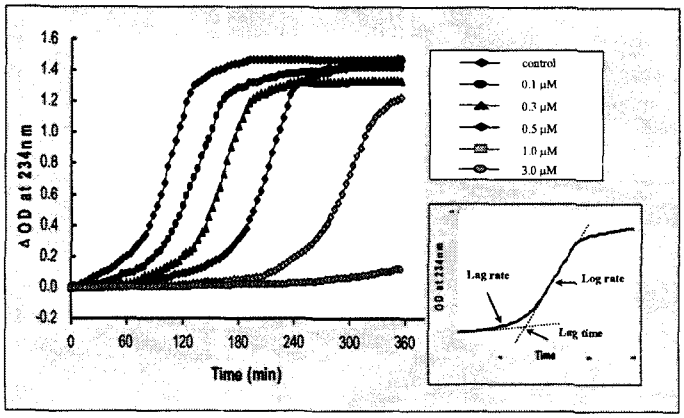
Inhibition of lipid peroxidation by *Melanocin A*



Lipid peroxidation was assessed by measuring the TBARS at 532 nm. Lipid peroxidation was initiated by the addition of 100 μM of FeSO4 into a mixture of 0.2mM ascorbic acid and 1.0 μg protein/ml microsomal suspension.

compound	rat liver microsomal lipid peroxidation (IC_{50}, μM)
Melanocin A	1.3
α-tocopherol	6.5
BHA	7.4

Inhibition of LDL oxidation by *Melanocin A*



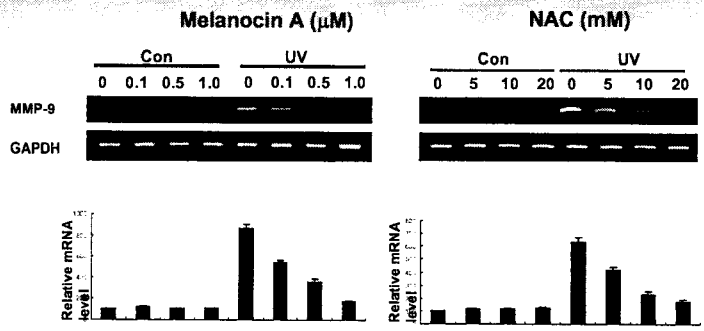
Inhibitory effects on tyrosinase and melanin formation

Inhibitory effects on mushroom tyrosinase and melanin formation in *Streptomyces bikiniensis* and B16 melanoma cells

Compound	*S. bikiniensis* NRRL-1049[a]	B16 Melanoma	Mushroom tyrosinase
	Inhibition zone(mm)	MIC[2](μM)	IC_{50}(μM)
Melanocin A	51	0.9	0.009
Melanocin B	0	-[3]	> 1mM
Melanocin C	0	-[3]	> 1mM
kojic acid	0	106.0	31.0
hydroquinone	25	-[3]	9.1
arbutin	-[3]	36.8	38.0
4-hydroxyanisole	30	-[3]	120.0

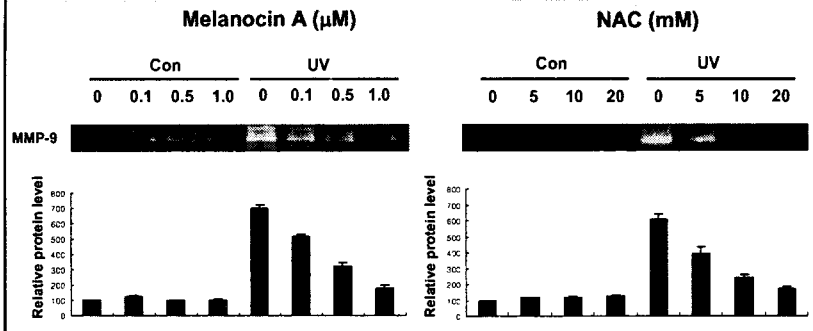
[a] Compound 30μg/ paper disc [2] Mininum inhibitory concentration [3] Not determined

Inhibition of UV-induced MMP-9 expression by Melanocin A in human keratinocytes (mRNA)



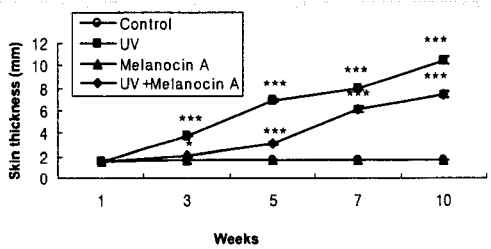
An immortalized human keratinocyte cell line, HaCaT cell, was serum starved for 48 h. After being pretreated for 1 h with Melanocin A or N-acetyl-cysteine (NAC), HaCaT cells were mock-treated or irradiated with UV (75mJ/cm2) in the presence of 1x PBS. Fresh media containing specific antioxidants were added and the cells were further incubated for 24 h. The expression levels of MMP-9 mRNA (A) were analyzed by semiquantitative RT-PCR. Levels of MMP-9 mRNA were normalized versus the corresponding GAPDH mRNA.

Inhibition of UV-induced MMP-9 expression by Melanocin A in human keratinocytes (Protein)



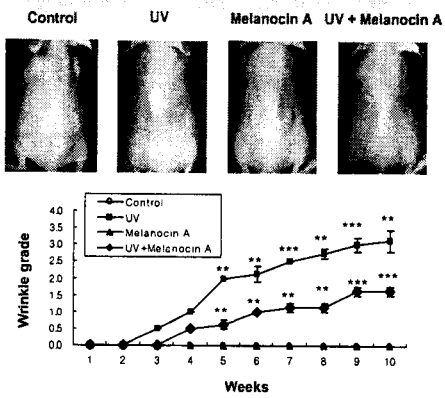
An immortalized human keratinocyte cell line, HaCaT cell, was serum starved for 48 h. After being pretreated for 1 h with Melanocin A or N-acetyl-cysteine (NAC), HaCaT cells were mock-treated or irradiated with UV (75mJ/cm2) in the presence of 1x PBS. Fresh media containing specific antioxidants were added and the cells were further incubated for 24 h. The amounts of MMP-9 protein (B) released into culture media were analyzed by zymography.

Inhibition of UV-induced skin thickness by Melanocin A in hairless mice



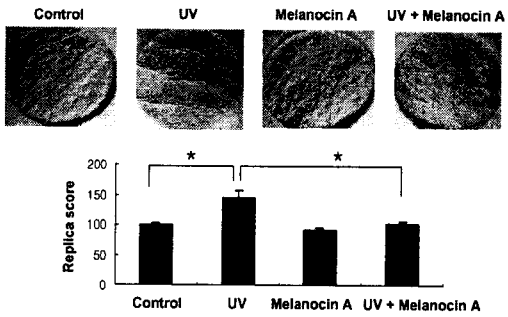
The dorsal skin surface of hairless mice were mock irradiated or irradiated with UV three times per week.UV dose were increased weekly by 1 MED (1 MED=130mJ/cm2) upto 4 MED and maintaine at this level for the remainder of the irradiation period. Vehicle only or melanocin A was applied to the backs of mice 2 hr before irradiation.

Inhibition of UV-induced wrinkle formation by Melanocin A in hairless mice



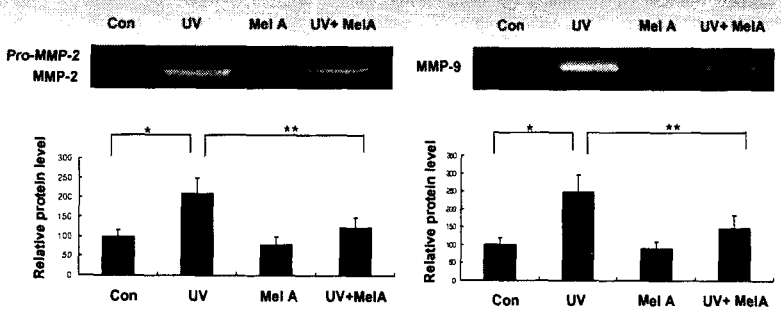
Wrinkle formation was measured by visual grading at the indicated times

Inhibition of UV-induced wrinkle formation by Melanocin A in hairless mice



Wrinkle formation was measured by silicon rubber replica grading at the indicated times. The skin impressions were photographed using stereomiscrope and analyzed using skinvisiometer SV600 software.

Inhibition of UV-induced MMP-2 and MMP-9 by Melanocin A in hairless mice



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UV dose were increased weekly by 1 MED (1 MED=130mJ/cm2) up to 4 MED and maintaine at this level for the remainder of the irradiation period.
Vehicle only or melanocin A was applied to the backs of mice 2 hr before irradiation.

Purification procedure and chemical structure of terrein from *Penicillum* sp.

***Penicillium* sp. KCTC26245**

↓ centrifuged at 3,000 rpm

Supernatant

↓ *extracted with ethyl acetate*

Ethyl acetate extract

↓

Sep-pack C18 column chromatography

↓

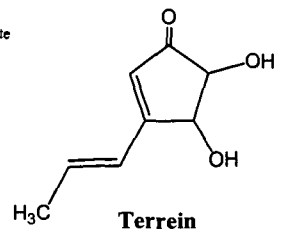
Sephadex LH-20 column chromatography

↓

Preparative HPLC

↓

Terrein



Terrein

Terrein(4,5-dihydroxy-3-propenyl-2-cyclopenten-1-one)

^{13}C NMR : 19.2(CH_3) , 78.0(CH), 82.5(CH), 125.0(CH), 126.1(CH), 145.0 (CH), 170 , 205.5 ppm
^{1}H-NMR : 1.94 ppm (3H, dd, J=6.9,1.5Hz), 4.07 ppm(1H, d, J=2.7Hz), 4.67 ppm (1H, d, J=2.7Hz), 6.0 ppm (1H, s), 6.44 ppm (1H, dd, J=15.6, 1.5), 6.81 ppm (1H, aq, J=6.9,15.6)

Effects of terrein on Mel-Ab cell viability

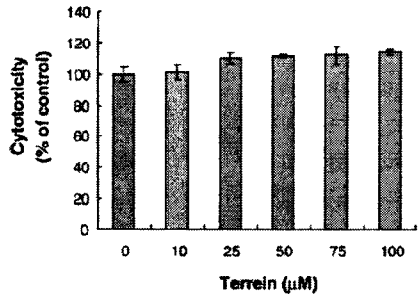


Figure 2. Effects of terrein on Mel-Ab cell viability. Cells were serum-starved for 24 h and terrein was added to serum-free medium at 10–100 μM for 24 h. Cell viabilities were determined by crystal violet assay. Each determination was made in triplicate and data shown are means ± SD.

Effects of terrein on Melanogenesis in Mel-Ab cells

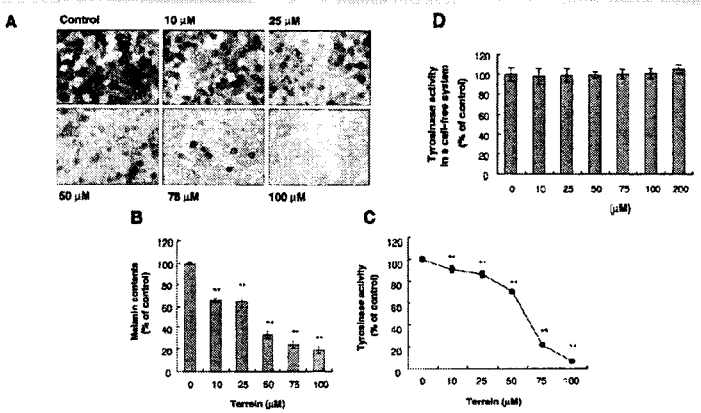
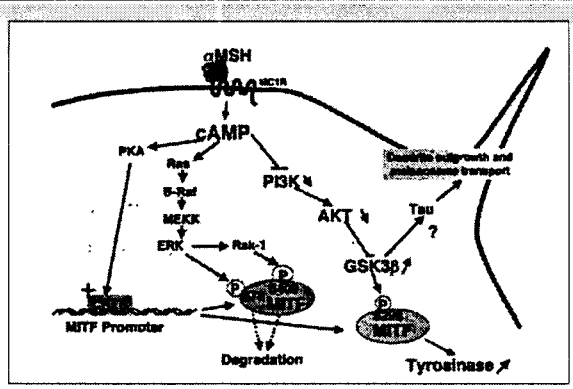


Figure 3. Effects of terrein on melanogenesis in Mel-Ab cells. Mel-Ab cells were cultured for 4 days in medium containing 10–100 μM terrein. Pictures were taken under a phase contrast microscope using a digital video camera (*A*). Melanin contents (*B*), tyrosinase activity (*C*) in Mel-Ab cells, and tyrosinase activity in a cell-free system (*D*) were measured as described in Materials and methods. The results are averages of triplicate experiments and the data shown represent means ± SD. ** $p < 0.01$ compared to the untreated control.

Signaling pathways involved in AMP-induced melanogenesis



ERK: extracellular signal-regulated protein kinase
MITF: microphthalmia-associated transcriptional factor
CREB: cAMP response binding-element protein

Terrein induces ERK activation, and down-regulates MITF

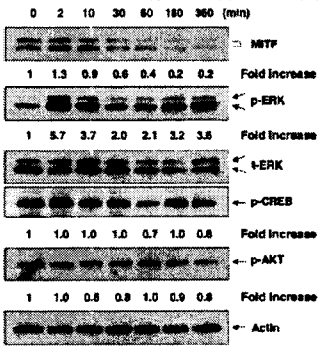


Figure 4. Terrein induces ERK activation, and down-regulates MITF. After 24 h of serum starvation, Mel-Ab cells were treated with terrein at the times indicated. Whole-cell lysates were then subjected to Western blot analysis using antibodies against MITF, phospho-specific ERK (p-ERK), phospho-specific CREB (p-CREB), and phospho-specific Akt (p-AKT). Equal protein loadings were confirmed using phosphorylation-independent ERK (t-ERK) or anti-actin antibody. Fold increases over the control were determined by densitometric analysis, and results are shown below each lane.

Terrein down-regulates MITF promoter activity

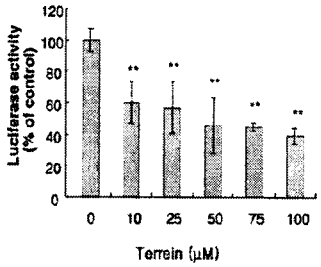


Figure 5. Terrein down-regulates MITF promoter activity. B16 cells were transfected with 2 μg of luciferase reporter plasmid and 1 μg pSV-β-galactosidase control vector. Cells were then incubated with terrein at 10–100 μM. Luciferase activities were measured as recommended by the manufacturer, and data were normalized with respect to β-galactosidase activity. Results are expressed as percentages of the untreated control. Each determination was made in triplicate and the data shown represent means ± SD. ** $p<0.01$ compared to the untreated control.

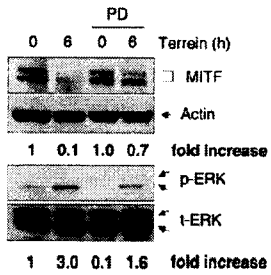
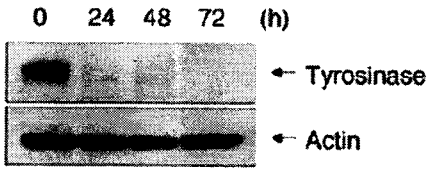


Figure 6. MITF down-regulation by terrein is correlated with ERK activation. Mel-Ab cells were starved in serum-free medium for 24 h, and then either pretreated or not with PD98059 for 1 h before terrein was applied for 6 h at 100 μM. Western blotting for MITF and phospho-specific ERK (p-ERK) was then performed with whole-cell lysates. Equal protein loadings were confirmed using phosphorylation-independent ERK (t-ERK) or anti-actin antibody. Fold increases over the control were determined by densitometric analysis, and results are shown below each lane.

Terrein decreases the protein level of tyrosinase in Mel-Ab cells



Cells were cultured with 100 uM of terrin for 24-72 h, and whole-cell lysates were then subjected to Western blot analysis with antibody against tyrosinase.

Acknowledgement

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