Novel Whitening Agent: Phytoclear-EL1

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Abstract

A novel melanogenic inhibitory compound, Phytoclear-EL1 (5,10-diacetyl-3-benzoyllathyrol) was isolated from Euphorbiae lathyridis. The effects of EL1 on cultured normal human melanocytes (NHM) were assessed. EL1 reduced the melanin synthesis of NHM by 40% at a concentration of 2 μg/ml without any apparent cytotoxicity. We also have found that the treatment of the cells with EL1 decreased the tyrosinase activity by 29% *in situ*. To elucidate the action mechanism of EL1, we investigated the changes in mRNA levels of tyrosinase, TRP-1 and TRP-2 using RT-PCR technique. As a result, the mRNA levels of tyrosinase, TRP-1 and TRP-2 were markedly reduced by EL1 treatment. These results suggest that Phytoclear-EL1 is a novel whitening agent that is effective in human melanocytes.

INTRODUCTION

The ambient levels of harmful ultraviolet (UV) radiation emitted by the sun are increasing due to destruction of the ozone layer. Therefore, our skin is exposed to more UV radiation and often suffers from various harmful effects of UV. Melanin pigmentation in human skin is a major defense mechanism against UV light of the sun, but abnormal hyperpigmentation such as freckles, chloasma, lentigines and other forms of melanin hyperpigmentation could be serious aesthetic problems (1). Therefore, potent active agents for the improvement of hyperpigmentation are sought for their cosmetic use. Many chemicals such as hydroquinone, arbutin, kojic acid, and ascorbic acid are well known for their melanogenic inhibitory functions (2, 3). Even though these chemicals are widely used as whitening agents in many cosmetic formulations and are melanogenic inhibitory in mouse and human melanoma cells, there were few demonstrative articles showing their effectiveness in cultured normal human melanocytes (NHM) and some controversies about their whitening effects exist (4, 5, 6). We have isolated 5,10-diacetyl-3-benzoyllathyrol (Phytoclear-EL1) from the crushed seeds of Euphorbiae lathyridis (*E. Lathyris*) and found that it exerted a strong

melanogenic inhibitory effect on B16 mouse melanoma cells. We also have found that it showed no apparent tyrosinase inhibition. In this study, we evaluated the effects of EL1 on cultured normal human melanocytes. In our experiment, the cell proliferation and melanin synthesis were evaluated by ^{3}H-thymidine and ^{14}C-DOPA incorporation, respectively. At a concentration of 2 □g/ml, EL1 markedly inhibited the melanin synthesis but did not affect the cell growth. The effect of EL1 on the tyrosinase activity of cultured normal human melanocytes was also investigated *in situ*. It reduced the tyrosinase activity in living cells. Because tyrosinase, TRP-1 and TRP-2 are thought to be the most important melanogenic enzymes, we examined the effect of EL1 on the expression of these enzymes. We found that EL1 reduced the mRNA levels of tyrosinase, TRP-1 and TRP-2. Our results clearly suggest that Phytoclear-EL1, in its action mechanism, is a novel whitening agent different from those ones, which are widely being used in cosmetic industry.

MATERIALS AND METHODS

Cell Cultures

B16 mouse melanoma cells were cultured in DMEM supplemented with 10% fetal calf serum in humidified incubator at 37℃ under 5% CO_2. Primary cultures of normal human melanocyte were established from neonatal foreskins. Cells were cultured in MCDB 153 (Sigma) medium supplemented with 1% fetal bovine serum (GIBCO BRL), 10 ng phorbol-12-myristate-13-acetate per ml (Sigma), 5 □g insulin per ml (Sigma), 0.5 □g hydrocortisone per ml, 1 ng basic human fibroblast growth factor per ml (Sigma), 10 nM alpha-melanocyte stimulating hormone (Sigma), 1 □g bovine transferrin per ml (ICN Biomedicals Inc), and antibiotic-antimycotic (GIBCO BRL). They were maintained in humidified incubator at 37℃ under 5% CO_2.

Effect of EL1 on Melanization in B16 Mouse Melanoma Cells

Cells were seeded into 60 mm dish at a density of 5×10^5 cells per dish. After cells were attached, medium was replaced with fresh medium (0.5% DMSO) containing various concentrations of EL1. Then cells were cultured for 2 days and the medium was replaced with fresh medium, further incubated for a day. Then cells were detached with cell scrapper, harvested, and counted with haemacytometer. Melanin was extracted and measured according to the method of Lotan with some modifications (7). Briefly,

cell pellets were resuspended in 1 ml of distilled water and freezed at -20℃ and thawed at 37℃. This freezing-thawing process was performed for three times. Perchlonic acid was added to the cell suspensions at a final concentration of 0.5N. The tubes were set on ice for 10 min and centrifuged at 15,000g for 5 min. The pellets were extracted with 0.5 N perchloric acid for 2 times, with cold ethanol/ether (3:1) for 2 times, and once with ether. The resulting pellets were dried in air and 1 ml of 1 N NaOH was added to each tube. The tubes were incubated in a boiling water bath for 10 min to dissolve the pellets. Melanin contents were measured by reading the absorbance at 400 nm and expressed as $A_{400}/10^6$ cells.

Measurement of Cell Growth and Melanin Synthesis in Normal Human Melanocytes
Cells were seeded into 24-well plate at a density of 2×10^4 cells per well and allowed to attach for 48 hrs. After then, two sets of triplicate cultures were fed with fresh medium containing EL1. After 48 hrs, the medium was replaced with the same, fresh medium and 1 □Ci of ^{3}H-methyl thymidine (Amersham Pharmacia Biotech) or 0.2 □Ci of ^{14}C-DOPA (Amersham Pharmacia Biotech).was added to each set of triplicate cultures, for assessing the cell growth and the melanin synthesis, respectively. The cells were further incubated for 48 h. After incubation, medium was discarded and the cells were rinsed with PBS, lysed by adding 0.5 ml of 1 N NaOH and incubating at 37℃ for 30min. The resulting cell lysates were transferred into liquid scintillation vials and neutralized with 0.1 ml of 5 N HCl, mixed with scintillation cocktail, and the radioactivity was determined by LS 6500 scintillation system (Beckman). We introduced a melanin index (MI, cpm value for ^{14}C / cpm value for ^{3}H), which could be used as a parameter for melanin contents per unit cell mass. Therefore, MI indicates the quantity of newly synthesized melanin for the defined amount of cell proliferation (DNA synthesis).

***In situ* Tyrosinase Assay**
Tyrosinase activity in living cells was assessed according to the method of Pomerantz with slight modifications (8). 2.5×10^5 normal human melanocytes were seeded into 60 mm dish and cultured for 2 days and the medium was replaced with fresh medium alone or medium containing EL1 and 1 □Ci/ml of L-[3,5-^{3}H] tyrosine, and further incubated for 24 hr. To measure ^3H_2O release, 1ml of culture medium was mixed with activated charcoal and incubated with agitation for 30 min at RT. The mixture was centrifuged 14,000 g for 10 min and 800 □l of supernatant was again mixed with activated charcoal, incubated, and centrifuged. 500 □l of resulting supernatant was taken into liquid scintillation vial, mixed with scintillation cocktail, and the radioactivity was determined

by LS 6500 scintillation system (Beckman, USA).

RNA Preparation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Normal human melanocytes were seeded into T-175 flask at a density of 0.5 x 10^6 cells per flask and allowed to attach for 48 hrs. After then, the medium was replaced with the fresh medium containing EL1. The cells were further incubated for 48 h. Total cellular RNA was prepared using RNAeasy Mini Kit (Qiagen) according to the supplier's instruction. Primers used for RT-PCR analysis in this study were as follows; tyrosinase (400bp), 5'TGCCAACGAT CCTATCTTCC3' (5'primer), 5'TGAGGAGTGGCTGCTTTTCT3'(3'primer); tyrosinase-related protein-1 (TRP-1, 450bp), 5'CCCTTGCGCTTCTTCAATAG3' (5'primer), 5'TTGCAACATTT CCTGCATGT3' (3'primer); tyrosinase-related protein-2 (TRP-2, 500bp), 5'CCGACTACGT GATCACCACA3' (5'primer), 5'TGGCAATTTCATGCTGTTTC3' (3'primer); glyceroaldehyde -3phosphate dehydrogenase (G3PDH, 752bp), 5'ATGTTCGTCATGGGTGTGAA'(5'primer), 5'GGGGTCTACAGGCAACTG3' (3'primer). These primers were synthesized by Bioneer co., Korea. For cDNA synthesis, 4 μg of the total RNA was reverse transcribed in 20 μl of reaction mixture containing 2 μl of 10x reverse transcription buffer (Perkin-Elmer), 4 μl of 25 mM $MgCl_2$ (Perkin-Elmer), 2 μl of 10 mM each dNTPs (Clontech), 1μl of 50 μM Oligo $d(T)_{16}$ (Perkin-Elmer), 20 units of RNasin (Promega) and 50 units of MuLV reverse transcriptase (Perkin-Elmer). Reverse transcription reaction mixture was incubated at RT for 10 min, 42°C for 15 min, 99°C for 5 min, and 5°C for 5 min (GeneAmp PCR system 2400 thermal cycler, Perkin-Elmer). For PCR amplification of cDNA, 1μl of the cDNA product was amplified in a total reaction volume of 50 μl containing 5 units of DNA Polymerase (AmpliTaq DNA Polymerase, Perkin-Elmer), GeneAmp 10 x PCR buffer (Perkin-Elmer), 4 μl of 25 mM $MgCl_2$ (Perkin-Elmer), 1μl of 10 mM each dNTPs (Clontech), 20 pmole upstream primer, and 20 pmole downstream primer. DNA amplification was performed using a Perkin Elmer Gene Amp PCR system 2400 thermal cycler. The PCR cycle conditions were melting for 15 seconds at 95℃, annealing for 30 seconds at 60℃, extension for 90 seconds at 72℃. PCR products were resolved on 1.8% agarose gel and visualized by ethidium bromide staining, photographed and analyzed withn Fluoro S multi-imager (Bio-Rad).

RESULTS AND DISCUSSION

Effect of Phytoclear-EL1 on Pigmentation B16 Mouse Melanoma Cells

EL1 showed the remarkable whitening effect on B16 melanoma cells. When cultured with 5μg/ml and 10μg/ml of EL1, there were 50% and 69% decrease in melanin contents in B16 melanoma cells, respectively (Fig. 1, A, B). These are very significant decrease in melanin contents when compared with other melanogenic inhibitors (data not shown).

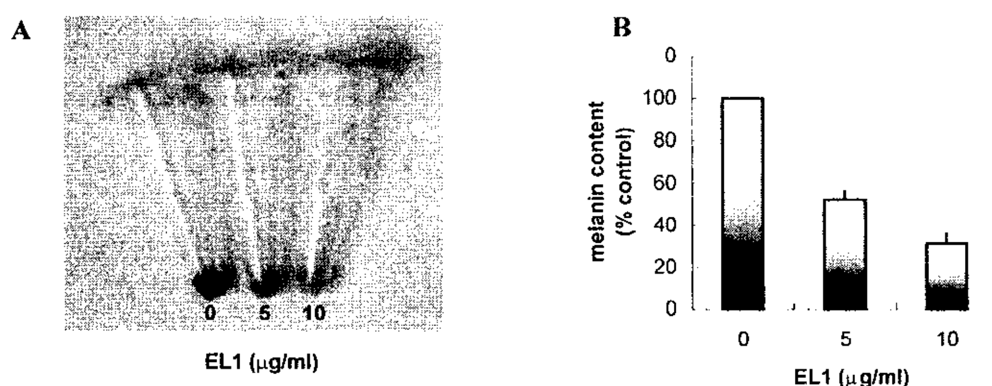

Figure 1. Phytoclear-EL1 decreased the pigmentation of B16 mouse melanoma cells. B16 F1 mouse melanoma cells were treated with or without EL1. After 3 days, cells were harvested. (A) Harvested cells were pelleted and photographed. (B) Their melanin contents were assayed as described in *Materials and Methods*. Melanin contents were expressed as percent of control. (n=3)

Effect of Phytoclear-EL1 on cell proliferation and melanin synthesis in Normal Human Melanocytes

The proliferation of human melanocytes treated with EL1 was evaluated by counting the incorporation of ^{3}H-methyl thymidine into the cells. Compared with untreated control, EL1 showed little inhibitory effect on cell proliferation at the tested concentration, 2 μg/ml (Fig. 2A).

We next examined the effect of EL1 on melanin synthesis by counting the incorporation of ^{14}C-DOPA into the cells. Although we found variation in the extent of inhibitory effects among the individual melanocyte strains, EL1 markedly reduced melanin synthesis (Fig. 2A). As a consequence, EL1 reduced the melanin index (MI: cpm value for ^{14}C/ cpm value for ^{3}H)) to 40% (Fig. 2B). This result shows that EL1 could inhibit

melanogenesis in human melanocyte but did not inhibit cell proliferation at proper concentration (2 μg/ml).

Effect of Phytoclear-EL1 on human tyrosinase activity *in situ*

Tyrosinase is the rate-limiting enzyme in melanin synthesis, and some melanin production-inhibiting agents such as arbutin and kojic acid are known to inhibit the tyrosinase activity. To determine the effect of EL1 on tyrosinase activity in living cells, we performed in situ tyrosinase assay. Compared with the untreated control, EL1 reduced total tyrosinase activity in normal human melanocytes by 29% at 2 μg/ml (Fig. 3). This is caused by two possibilities. One is inhibition of tyrosinases and the other is the reduction of tyrosinase synthesis. Because EL1 showed no inhibition on mushroom tyrosinase, we have ruled out the first one (data not shown). So, we had an idea that the reduced amount of tyrosinase was a main cause of this phenomenon.

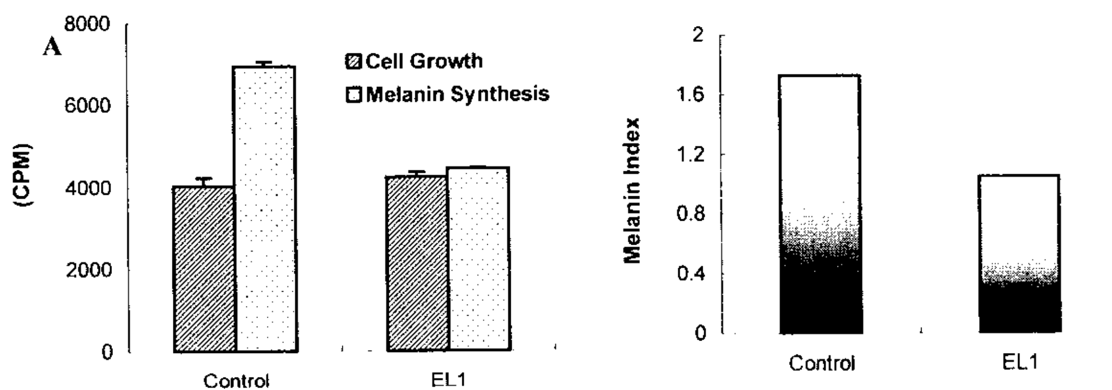

Figure 2. Phytoclear-EL1 inhibited the melanin synthesis of normal human melanocytes. Melanocytes were seeded into 24-well plate at 2 X 10^4 cells per well and treated with or without EL1 2 μg/ml for 4 days. For the last 48 h treatment, the medium was replaced with fresh medium supplemented with 1 μCi of ^{3}H-methyl thymidine or 0.2 μCi of ^{14}C-DOPA for assessing the cell growth and the melanin synthesis, respectively. Melanin Index (MI) is equal to {(cpm value for ^{14}C) / (cpm value for ^{3}H)}. (A) Cell growth and melanin synthesis. (B) Melanin index. Values are the averages of three determinations ± SD.

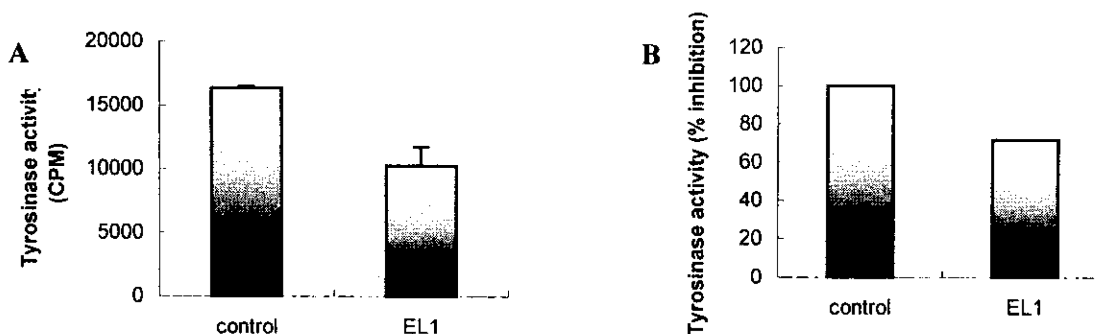

Figure 3. Phytoclear-EL1 inhibited human tyrosinase activity.
$2.5x10^5$ normal human melanocytes were seeded into 60 mm dish and cultured for 2 days and the medium was replaced with fresh medium containing 1 μCi/ml of ^3H tyrosine and further incubated for 24 hr. At the end of incubation, tyrosinase activities were determined as described in *Materials and Methods.* Control (0.1% DMSO), EL1 (2 μg/ml in 0.1% DMSO). Changes in tyrosinase activity (A) and the degree of inhibition is expressed as % of control (B). Values are the averages of three determinations ± SD.

Effect of Phytoclear-EL1 on expression of tyrosinase, TRP-1 and TRP-2 genes

To explore the mechanism responsible for the decreased pigmentation caused by EL1, we also investigated the changes in the mRNA levels of three important melanogenic enzymes (tyrosinase, TRP-1 and TRP-2) using RT-PCR. Human melanocytes were treated with 2 μg/ml of EL1 for 4 days and then, each mRNA level was examined. Fig. 4A shows the changes in mRNA levels in EL1 treated cells. When normalized with G3PDH mRNA level, the mRNA levels of tyrosinase, TRP-1 and TRP-2 were decreased by 42%, 45% and 24%, respectively, as compared to untreated control (Fig 4B). These results suggest that EL1 might act on the common upstream event that controls the transcription of three melanogenic genes.

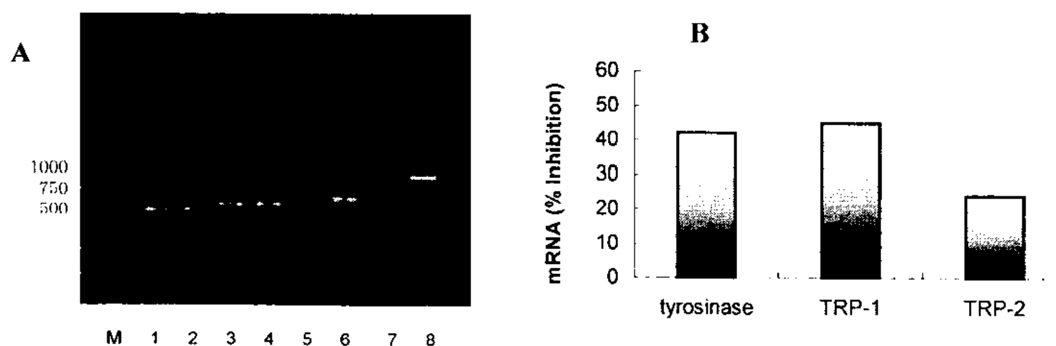

Figure 4. **Phytoclear-EL1 decreased expression of tyrosinase, TRP-1 and TRP-2 genes.** Normal human melanocytes were seeded into T-175 flask at a density of 0.5 x 10^6 cells per flask and treated with or without EL1 2 μg/ml for 4 days. Total RNA was extracted and applied to RT-PCR as described in *Materials and Methods.* (A) Gel electrophoresis of the RT-PCR products. M: size marker, Lane 1, 3, 5, 7: PCR product of tyrosinase (400bp), TRP-1 (450bp), TRP-2 (500bp) and G3PDH (752bp) of untreated control, respectively; Lane 2, 4, 6, 8: PCR product of tyrosinase, TRP-1, TRP-2 and G3PDH of EL1-treated NHM, respectively. (B) When EL1 was treated, the decreased mRNA level was expressed as % inhibition.

CONCLUSIONS

We have concluded that;

1. Phytoclear-EL1 (5,10-diacetyl-3-benzoyllathyrol), a novel whitening agent, was isolated from Euphorbiae lathyridis.
2. Phytoclear-EL1 inhibited melanin synthesis not only in B16 mouse melanoma cells but also in cultured normal human melanocytes.
3. Phytoclear-EL1 exerts its melanogenic inhibitory effect through the modulation of mRNA levels of tyrosinase, TRP-1, and TRP-2.

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