

Novel anti-melanogenic hexapeptoids, PAL-10 and PAL-12

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Abstract Diverse compound sources are being explored for de-pigmentation activities to develop novel therapeutic agents or functional cosmetic ingredients for hyper-pigmentation disorders. Peptoids are a class of peptidomimetics whose side chains are appended to the nitrogen atom of the peptide backbone, instead of α -carbon. Peptoids are more durable against proteolysis and are being actively investigated in drug discovery, but rarely studied as cosmetic ingredients. Here, we demonstrated that new hexa-peptoids, PAL-10 and PAL-12, can inhibit melanogenesis in B16F10 melanoma cells, a 3D pigmented human skin model (Neoderm[®]-ME, Tegoscience Co) and zebrafish. Anti-melanogenic effects of PAL-10 or PAL-12 as compared with arbutin, a positive control in B16F10 cells, Neoderm[®]-ME and zebrafish were statistically

significant and concentration-dependent anti-melanogenic effects were manifested as determined by image, histology, and melanin contents. Anti-melanogenic effects of PAL-10 appeared to be from enzymatic inhibition of tyrosinase while mRNA expression of melanogenic enzymes was not affected. In conclusion, we demonstrated that PAL-10 and PAL-12 can be used as a new cosmetic ingredient with strong brightening efficacies.

Keywords Peptoid · Melanogenesis · Zebrafish · 3D human skin models · Tyrosinase

Introduction

Hyperpigmentation disorders like post-inflammatory hyperpigmentation, melasma, freckles (or ephelides), and dark spots remain major dermatological and cosmetical problems [31]. Many de-pigmenting ingredients like bisabolol [8], arbutin, kojic acid, and hydroquinone [2] have been developed but there is still a large demand for new, safer and more active de-pigmenting agents. Indeed, to discover novel de-pigmenting agents, various compound sources like natural products [4], endogenous or engineered peptides [17, 18, 27], and synthesized chemicals [10, 11] are being actively explored.

Oligopeptides or short sequence amino acids are garnering recent attention for therapeutic use in dermatological diseases and as functional cosmetic ingredients. Benefits of a peptide fragment [decapeptide-12, Tyr-Arg-Ser-Arg-Lys-Tyr-Ser-Ser-Trp-Tyr, a synthetic fragment of basic FGF (Fibroblast Growth Factor)] for hyper-pigmentation disorders have been demonstrated in clinical trials [7, 17], reflecting their utility as depigmentating agents. Despite potent biological activities, however, oligopeptides are rapidly

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cleared through enzymatic degradation limiting their duration of action substantially. To overcome this limitation, it has become a common practice in drug discovery to structurally modify or derivatize peptides to prevent proteolytic degradation [30]. There also have been some attempts to develop cosmetically active and bio-durable peptide derivatives for the use as cosmetic ingredients [12, 19].

Peptoid is a peptide mimetic in which the side alkyl chain is linked to the nitrogen of the amide backbone instead of the α -carbon in peptides (Fig. 1a) [24]. Due to the

absence of free amide linkage (substituted amine), peptoid is resistant to protease-mediated degradation and accordingly, more durable than natural peptides in viable tissues [9, 14]. This feature is beneficial for the manifestation of efficacy of cosmetic ingredients since protease systems are well developed within the skin [6, 13] and can rapidly degrade the active ingredient. In addition, using primary amine of various functional groups, a large chemical diversity can be harbored in peptoid or peptide-peptoid hybrid sequences than natural peptides [5]. Numerous efforts have been made to discover novel drug candidates [33, 34] and biopolymers [16] employing peptoid sequences in the fields of anti-cancer, analgesic, and antibacterial drugs but little is known about the utility of peptoids as new de-pigmenting agents to our best knowledge.

Here, through screening of 50 palmitoyl peptoids that were basically N-derivatized peptoids on a palmitoyl hexapeptide sequence (Val-Gly-Val-Ala-Pro-Gly), a repeating peptide in elastin which showed chemotactic activities for fibroblasts and monocytes [23], and proliferative activities in smooth muscle cells [28], we discovered new hexa-peptoids, PAL-10 and PAL-12 (Fig. 1b), with strong de-pigmenting activities. PAL-Val-Gly-Val-Ala-Pro-Gly has been successfully applied as a cosmetically active ingredient [20]. Linking of palmitoyl-chain to peptide or peptide mimetic is known to enhance its skin permeation prominently [17, 26]. PAL-10 is a peptoid analog of the elastin hexapeptide sequence where the terminal N atom was linked to tryptamine. In case of PAL-12, isobutyl was linked to the terminal N atom. For the evaluation of the effects of PAL-10 and PAL-12 on pigmentation, diverse experimental systems have been employed like murine melanoma cell line, B16F10, 3D reconstructed human epidermis model, Neoderm[®]-ME, and zebrafish model where potent de-pigmenting activities of PAL-10 could be demonstrated. We also explored the mechanism underlying the anti-melanogenic effect of PAL-10 through examining enzymatic expression and activity inhibition. With this study, we believe that important clue has been provided for novel utility of peptoids as functional cosmetic ingredients.

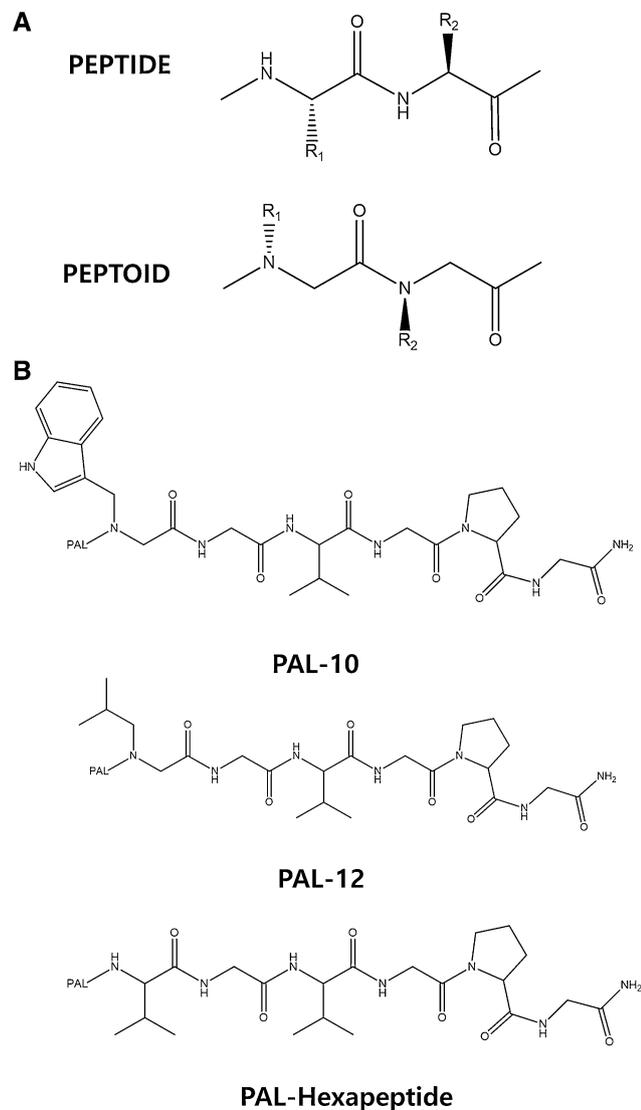


Fig. 1 Structures of peptide, peptoid, PAL-10, PAL-12, and PAL-hexapeptide. **a** Structure of peptide and peptoid. **b** Structure of PAL-10, PAL-12, and PAL-hexapeptide. PAL-10 and PAL-12 are peptoids where terminal amino acid residue is replaced by peptoid sequences. PAL-10 is a peptoid analog of elastin hexapeptide sequence where the terminal N atom was linked to tryptamine while isobutyl is linked in case of PAL-12. PAL-hexapeptide is a palmitoylated elastin hexapeptide sequence. Rn is alkyl group. Different number indicates different alkyl group

Materials and methods

Materials

Peptoids, PAL-10 and PAL-12, were synthesized by AnPEP(Ochang, Chung-buk, Korea) as depicted in Fig. 2a. Chemicals for synthesis, arbutin, mushroom tyrosinase, α -melanocyte stimulating hormone (α -MSH), DMSO, salts, and buffering agents were from Sigma-Aldrich (St Louis, MO., USA). Cell culture medium and agents were from Thermo Scientific (Waltham, MA., USA).

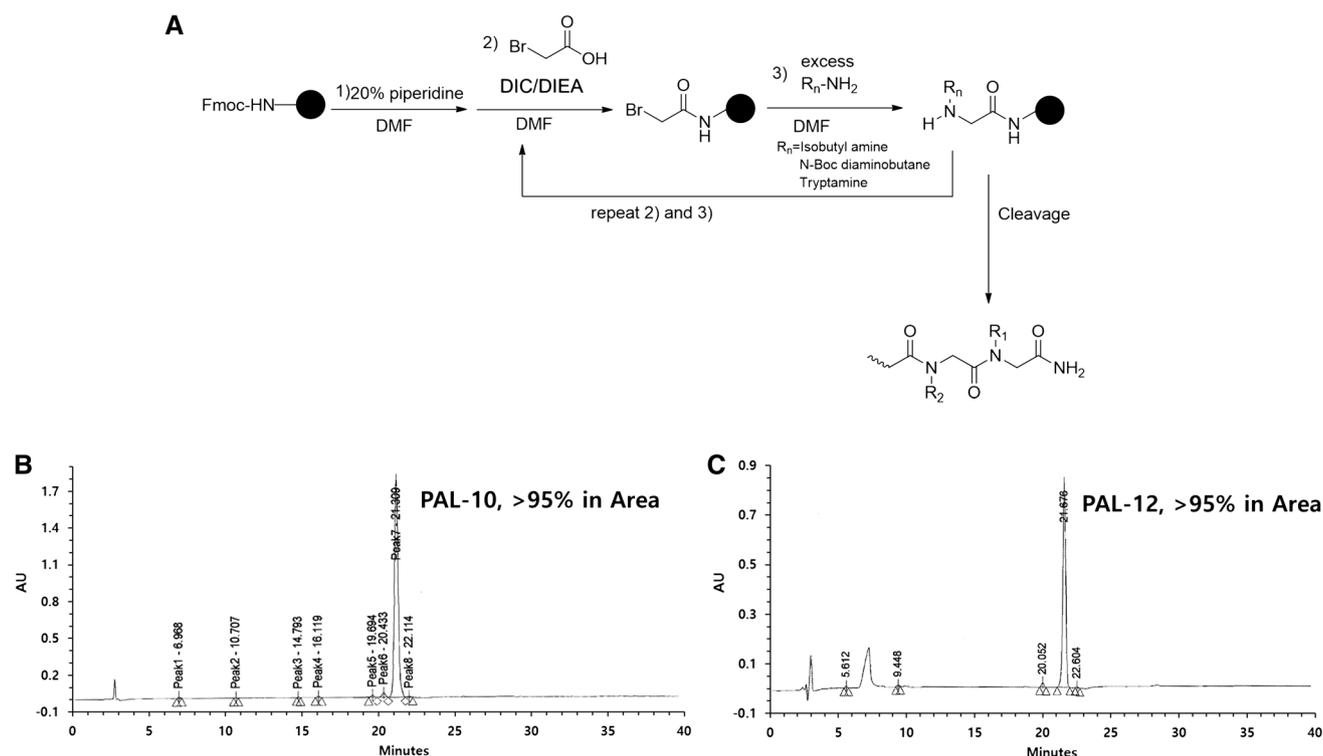


Fig. 2 Effects of PAL-10 and PAL-12 on the melanin synthesis in B16F10 cells. **a** Synthetic scheme for the preparation of peptoids. For more details, see “Materials and Methods”. **b** Purity of PAL-10 and PAL-12 (>95 %) checked by HPLC chromatogram. R_n is alkyl

group. Different number indicates different alkyl group. Fmoc (9-fluorenylmethoxycarbonyl), DMF (dimethylformamide), DIC (*N,N'*-diisopropylcarbodiimide), and DIEA (*N,N'*-diisopropylethylamine)

Preparation of peptoids

After swelling of solid-state Rink Amide resin (0.61 mmol/g) in dimethylformamide (DMF) for 20 min, 20 % 9-fluorenylmethoxycarbonyl (Fmoc) group was removed with piperidine/DMF. Then, resin was washed with DMF three times and underwent reaction with bromoacetic acid (20 eq), *N,N'*-diisopropylcarbodiimide (DIC, 20 eq), and *N,N'*-diisopropylethylamine (DIEA, 20 eq) for 90 min. This step was repeated twice after which a primary amine (30 eq) was added and underwent reaction for 12 h. Resultant peptoid was cleaved from resin in trifluoroacetic acid/H₂O/triisopropylsilane = 90:5:5(v/v/v). Crude peptoid was collected by recrystallization with ether and further purified and the final purity was checked (Fig. 2a, b) employing HPLC (2695 Alliance, Waters Co., Milford, MA., USA). Identity of peptoid was checked with mass spectrometry (MALDI-TOF/TOFMS, Bruker Daltonics, Bremen, Germany).

Cell culture and evaluation of melanogenesis

B16F10 mouse melanoma cells (ATCC, Manassas, VA., USA) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing of 10 % fetal bovine

serum(FBS)1 % L-glutamine, and 100 units/ml penicillin/streptomycin. One day prior to an experiment, cells (1 × 10⁴ cells) were seeded into 48-well plate for overnight. For the measurement of melanin contents, cells were treated with indicated compound and 100 nM α -MSH or irradiated with UVB-dose 15 mJ/cm² from the UV-source (BIOSUN, VilberLourmat, Cedex, France) and further incubated for 72 h. After media was removed and cells were washed twice with phosphate-buffered saline and dissolved in 100 μ L of 1 N NaOH at 60 °C for 1 hr in the dark. The total melanin content was estimated by absorbance at 405 nm (Spectra max 190, Molecular Devices, Sunnyvale, CA., USA). Morphology of B16F10 was observed with microscope (X400, ECLIPSE TS100, Nikon, Tokyo, Japan).

Effects of on skin-whitening using the pigmented 3D human epidermis model, Neoderm[®]-ME

Neoderm[®]-ME (Tegoscience, Seoul, Korea) is a pigmented 3D human epidermis model in which human primary keratinocytes and melanocytes are 3-dimensionally cultured to mimic the morphology and physiology of human skin. Briefly, Normal human keratinocytes from an established

cell bank were seeded onto collagen gels containing fibroblasts. After 5 days, cultures were raised to the air-liquid interface to allow the keratinocytes to differentiate and maintained for 14 days. Melanocytes were also seeded with keratinocytes for Neoderm[®]-ME. The Neoderm[®]-ME was incubated with α -MSH and incubated for 3 days and then irradiated with UVB doses of 60 mJ/cm². UVB-irradiated Neoderm[®]-ME was post-incubated for 3 days and treated with the compounds. Melanin content was measured at 405 nm from the tissue extract, dissolved with 1 N NaOH. Fontana-Masson staining was used for medium disposition from the paraffin-sectioned medium. Fontana-Masson staining was adapted from a method described previously [32].

Effects on melanogenesis in zebrafish of in vivo lightening model

Zebrafish (*DanioRerio*) was grown as previously reported [3]. Synchronized embryos of zebrafish were treated with melanogenic inhibitors at the indicated concentrations. The compound were dissolved in 0.1 % DMSO and then added to the embryo medium. After 72 h of incubation, the effects on the pigmentation of zebrafish were observed under microscope (ECLIPSE TiU, Nikon, Tokyo, Japan) and the intensity was analyzed using image analysis software (NIS-Br, ver. 4.07, Nikon).

In vitro mushroom tyrosinase activity assay

A cell-free assay system was used to investigate the direct effects of anti-melanogenic compounds on tyrosinase activity. Briefly, 24 μ L of phosphate buffer containing compound was mixed with 12.5 units of mushroom tyrosinase, and 180 μ L of 0.03 % tyrosine in deionized water was added. After incubation at 37 °C for 10 min, absorbance was measured at 475 nm (Spectra max 190, Molecular Devices, Sunnyvale, CA, USA).

Real-time PCR

To determine the effects of PAL-10 and peptoid-12 on melanogenesis-related gene expression, real-time transcription-polymerase chain reactions were used. B16F10 cells were treated with or without PAL-10 with α -MSH. After 24 h of incubation, total RNA was isolated using RNeasy plus mini kit (Qiagen, Valencica, CA., USA). For analysis of the tyrosinase mRNA levels, cDNA amplification was carried out with 1,250 ng of total RNA with oligodT (Bioelipsis, Seoul, Korea) and qPCR machine (Applied Biosystem, Grand Island, NY., USA). The sequences of

PCR primers were: tyrosinase 5'-g g gc cc a aa tt g t a c a g a g a-3' (forward) and 5'-a t g gg t g t t g a c cc a t t g t t-3' (reverse), TRP-1 5'-g t t c a a t g g c c a g g t c a g g a-3' (forward) and 5'-c a g a c a a g a a g c a a c c c c g a-3' (reverse), and TRP-2 5'-g c t t g g a g c a g c a a g a c a a g-3' (forward) and 5'-a t t a c a c a g t g t g a c c c g g c-3' (reverse). Cycling parameters were 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C 15 s, and 50 °C 1 min.

Statistics

Data are expressed as mean \pm standard error of mean (S.E.M.) of three or more independent experiments to analyse statistical difference from the control group [15]. The statistical analyses were performed by student *t* test. A *p* value <0.05 was considered statistically significant.

Results

Inhibition of melanin synthesis in murine melanocytes by PAL-10 and PAL-12

Effects of PAL-10 or PAL-12 on melanin synthesis were evaluated in murine melanoma cells. As shown in Fig. 3a and b, melanogenesis was significantly and potently inhibited by PAL-10 and PAL-12 in B16F10 cells in dose-dependent manner. Consistently with this effect, α -MSH-induced elongation of dendrites of melanocytes were attenuated by the treatment of PAL-10 and PAL-12 (Fig. 3c). Furthermore, we exposed the cell to 15 mJ/cm² UVB to confirm the anti-melanogenic effects of PAL-10 and PAL-12. As shown in Fig. 3d, PAL-10 at 50 μ g/mL inhibited melanin synthesis significantly while PAL-12 failed to attenuate UVB-induced melanogenesis.

Whitening effects of PAL-10 and PAL-12 in the pigmented 3D human epidermis model, Neoderm[®]-ME

We examined the effectiveness of PAL-10 and PAL-12 in skin-whitening using the pigmented 3D human skin epidermis model (Neoderm[®]-ME). Neoderm[®]-ME was stimulated with α -MSH, incubated for 3 days, and then irradiated with UVB (60 mJ/cm²). After post-incubation for 3 days, tissues were treated with PAL-10, PAL-12 or a positive control, arbutin. Figure 4a and b indicates dose-dependent inhibition of melanin content in Neoderm[®]-ME following the treatment of PAL-10 and PAL-12. Consistently with UVB-induced melanogenesis assay in B16F10 cells, PAL-10 was more effective than PAL-12 in the attenuation of melanogenesis in Neoderm[®]-ME. In addition,

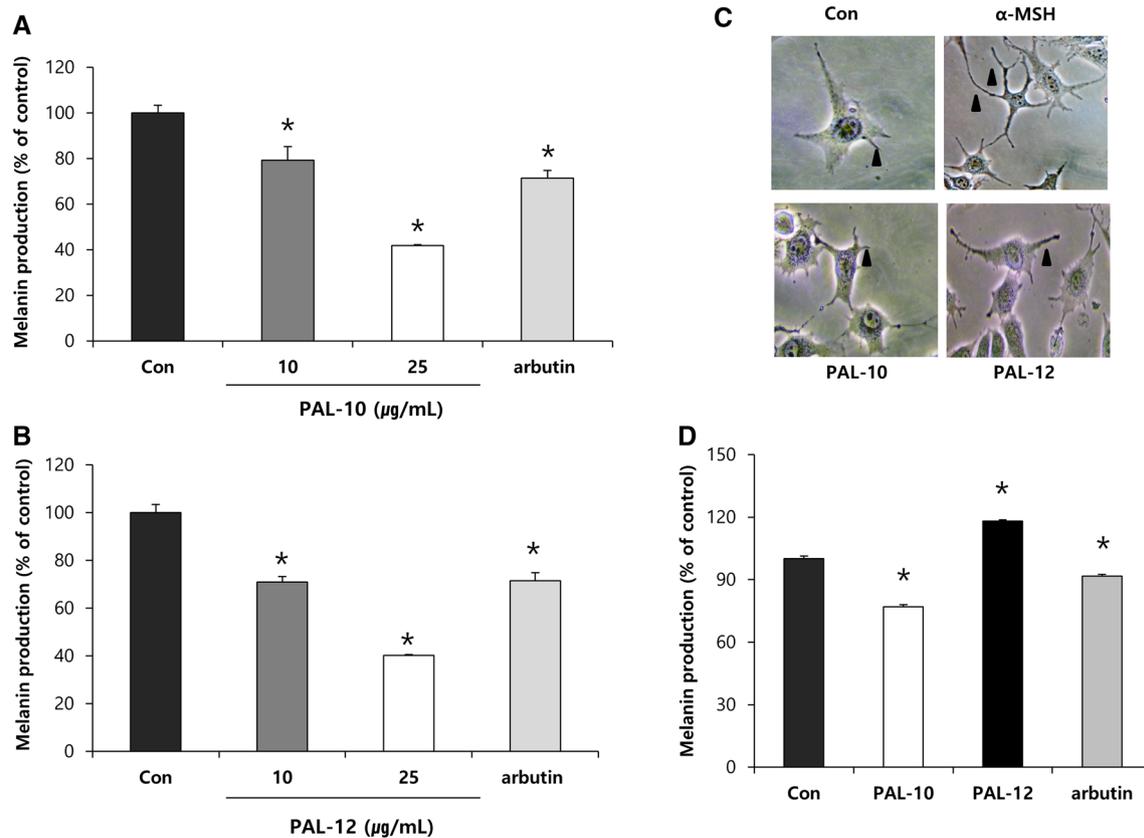


Fig. 3 Effects of PAL-10 and PAL-12 on the melanin synthesis in B16F10 cells. B16F10 cells were treated with various concentrations of PAL-10 and PAL-12 (10 and 25 μ g/mL) along with α -MSH. **a**, **b** Measurement of melanin production. **c** Microphotographs for morphological change ($\times 400$). *Arrowhead* indicates dendrite

formation from melanoma cells. **d** B16F10 cells were treated with PAL-10 and PAL-12 (50 μ g/mL) for 72 h after the exposure of 15 mJ/cm² UVB. Positive control was 25 μ g/mL arbutin. *Asterisk* represents statistical difference from control. $N = 3-4$

Neoderm[®]-ME was stained with Fontana-Masson to detect melanin distribution. Photograph shows the reduction of melanin distribution in the tissues treated with 100 μ g/mL PAL-10 or arbutin (Fig. 4c), confirming the whitening effects of PAL-10.

Effects of PAL-10 on the pigmentation of zebrafish in vivo

Zebrafish model is an in vivo melanogenesis model in which systemic toxicity as well as whitening effects can be evaluated [3]. Synchronized embryos of zebrafish were treated with PAL-10 or arbutin at indicated concentrations. After 72 h of incubation, the effects on the pigmentation of zebrafish were examined under microscope. Treatment of PAL-10 or arbutin did not affect the viability or activity of zebrafish (data not shown). As shown in Fig. 5a and b, the brightness of zebra fishes was increased dose-dependently by PAL-10 treatment. Especially, whitening effects of PAL-10 0.1 μ g/mL were almost equivalent to those of 10 μ g/mL arbutin.

Effects of PAL-10 activity of tyrosinase and the expression of melanogenic enzymes

To explore the mechanism of de-pigmenting activities of PAL-10, we measured the effects of PAL-10 on the tyrosinase activity using cell-free mushroom tyrosinase system. As shown in Fig. 6a, tyrosinase activities were inhibited in a concentration-dependent manner by PAL-10 treatment. In contrast, expression of tyrosinase, TRP-1 or TRP-2 was not suppressed by PAL-10 (Fig. 6b), indicating that PAL-10 has no effect on the transcriptional regulation of melanogenic enzymes.

Discussion

Here, we demonstrated novel anti-melanogenic effects of hexapeptoids, PAL-10, and PAL-12 employing B16F10 murine melanoma cell line, a pigmented 3D reconstructed human epidermis model, NeodermME[®], and zebrafish model in vivo. Especially, PAL-10 was broadly effective in attenuating melanogenesis in vitro and in vivo at the

Fig. 4 PAL-10 induces skin-whitening in the pigmented 3D human skin epidermis model, Neoderm[®]-ME. Neoderm[®]-ME was treated with various concentrations of PAL-10, PAL-12 (50, 250 μg/mL). **a**, **b** Measurement of melanin production. **c** Melanin pigment on the 3D skin model was visualized by Fontana-Masson staining. Positive control was arbutin 1 mM

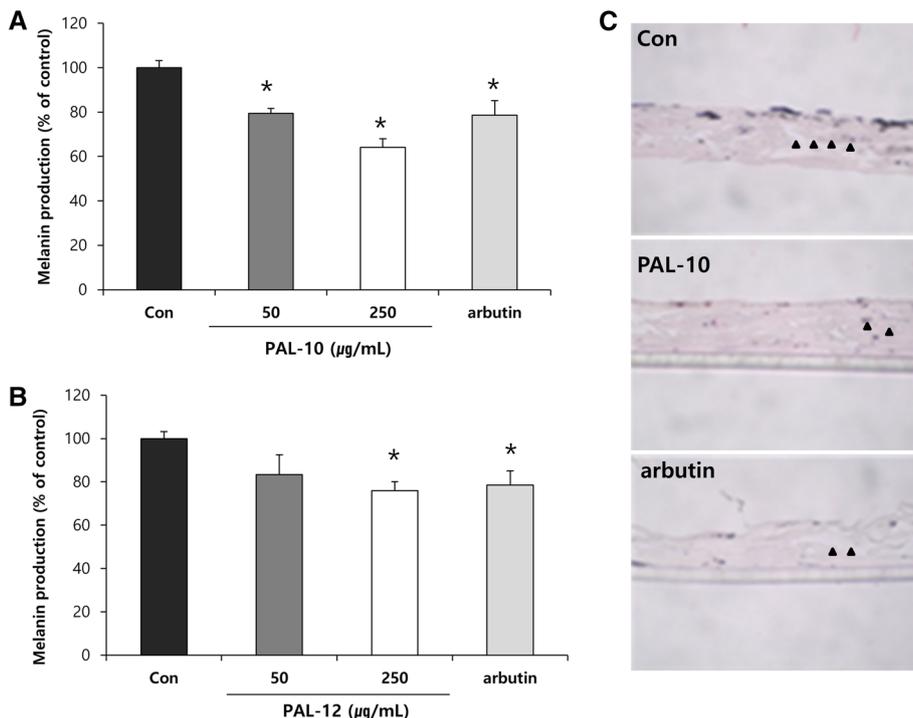
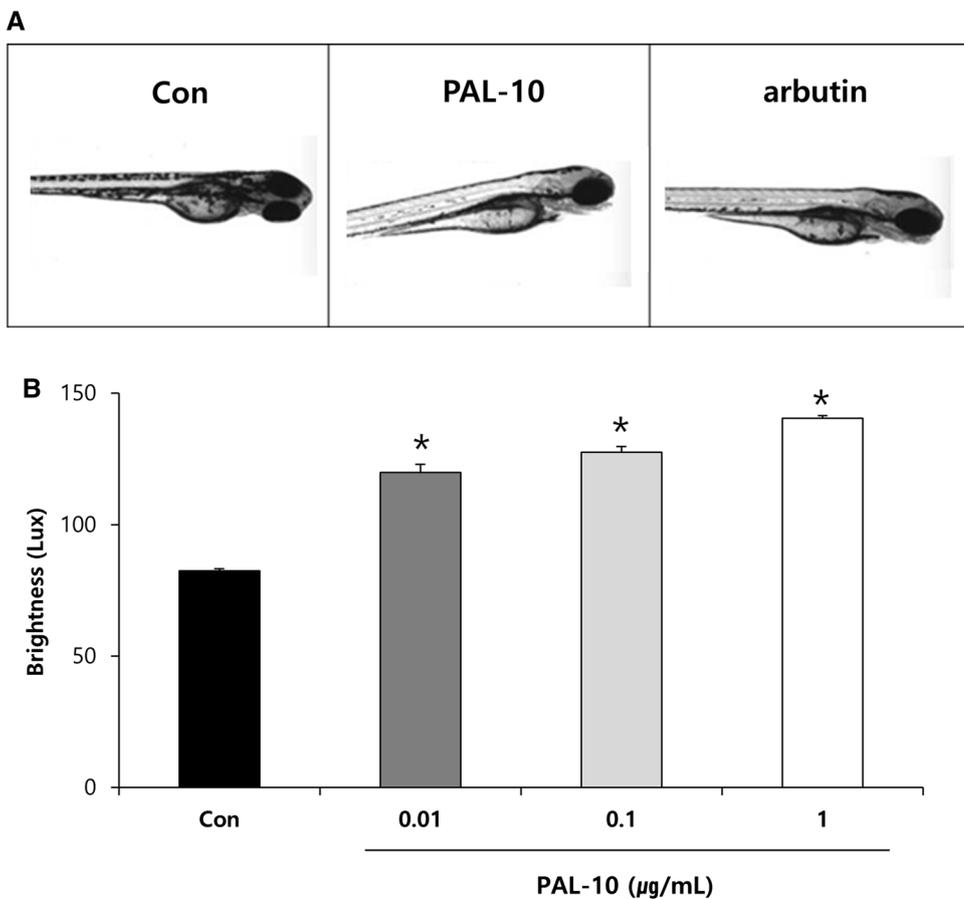


Fig. 5 Effects of PAL-10 on the pigmentation of zebrafish. Zebrafish embryos were treated with PAL-10 at the indicated concentrations through adding to the medium. The effects on the pigmentation of zebrafishes were examined by **a** a stereomicroscope and **b** by measuring *brightness*. Positive control, arbutin was 10 μg/mL



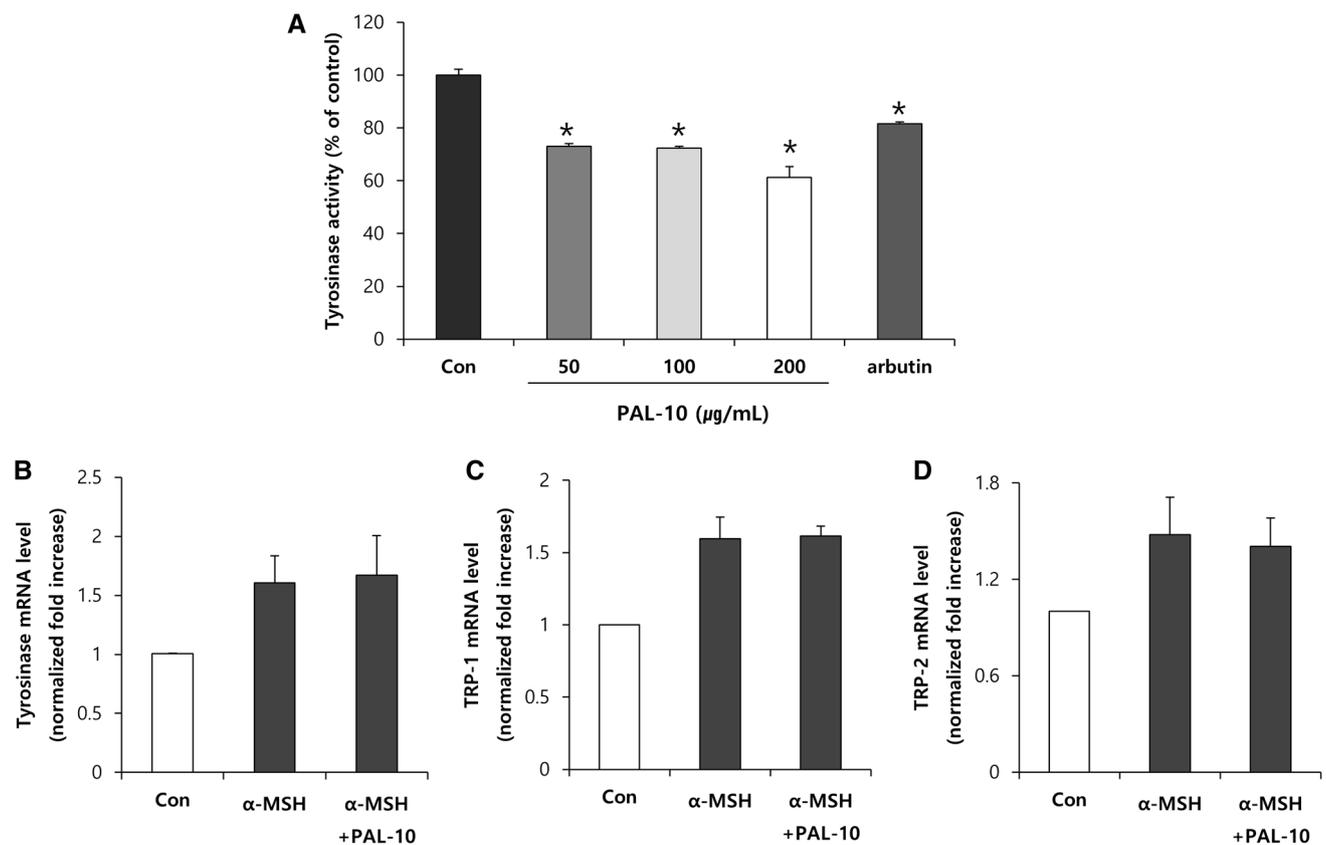


Fig. 6 Effects of PAL-10 on mushroom tyrosinase activity and expression of melanogenic enzymes. **a** Effects of PAL-10 on mushroom tyrosinase were measured using L-tyrosine. **b** mRNA level

of tyrosinase, TRP-1 and TRP-2 were assessed by real-time PCR in B16F10 cells after treatment of PAL-10 (50 µg/mL)

concentration ranges from 0.01 µg/mL in zebrafish model to 50 µg/mL in 3D reconstructed human epidermis model, demonstrating its strong potency and a high therapeutic potential as a novel de-pigmenting agent.

Peptides are susceptible to proteolytic degradation. Exopeptidases that degrade terminal residue of peptides are reported to substantially contribute to the degradation of bioactive peptides in viable tissues, markedly curtailing the tissue-resident time for the manifestation of activities. This is especially true in skin epidermis where diverse protease systems like serine-protease, matrix metalloproteinases, and matriptase orprostin are well-developed and closely associated with skin physiology [13]. In this regard, PAL-10 and PAL-12, peptoid-modified palmitoylhexa peptides may be good alternatives that are resistant to the protease-mediated degradation through applying multiple peptide derivatization, i.e. palmitoylation and peptoid on the terminal valine residue, allowing longer time to manifest efficacy in vivo. Supporting this, PAL-10 was effective in whitening zebrafish and pigmented 3D reconstructed human epidermis model where the activity of proteases are well-preserved, reflecting that PAL-10 might be effective in human although further clinical trials are necessary to prove it.

Diverse models for the assessment of melanogenesis have been employed in the present study. Each model has respective advantages and disadvantages. B16F10 model is cheap and quick to evaluate de-pigmentation efficacy allowing high throughput assay but difference in post-translational modification of tyrosinases from that of human and absence of metabolic activity (which is enriched in epidermal layer) may overestimate or underestimate the de-pigmenting efficacies, requiring further confirmation steps in human melanocyte and metabolically competent models. Zebrafish model is recently introduced in vivo model for melanogenesis [1, 3]. This model has numerous merits including capacity to evaluate in vivo activities, easy maintenance, high drug penetration and most importantly, liberty from animal test issues in evaluating cosmetics. In addition, this model can give important clues to potential systemic toxicity, which has become increasingly important due to the recent issues involving toxicity of a de-pigmenting agent, rhododendol [21]. Pigmented 3D human epidermis model is also garnering a huge popularity [25, 29, 31] owing to the employment of human primary melanocytes and existence of metabolically competent epidermal layer. In this model, anti-melanogenic effects on

human melanocytes can be evaluated in near in vivo condition. However, zebrafish and 3D human epidermis models are relatively expensive and low in throughput as compared to B16F10 cell line model, suggesting that tiered and systemic use of diverse anti-melanogenic models is more efficient for discovery of new effective and safe depigmenting agents.

Anti-melanogenic activities of PAL-10 appeared from direct enzymatic activities of tyrosinase but not from the modulation of expression of melanogenic enzymes. Incidentally, anti-tyrosinase activities of peptides were reported previously [22]. Tyrosinase inhibitory activities can be found in the peptides containing arginine, Phe, Val, Ala or Leu in their sequences. PAL-10 contains two Val and Ala in its sequence which may explain its inhibitory activities. In addition, PAL-10 was effective in attenuating α -MSH-induced and UV-induced melanogenesis, which can be explained by the inhibition of a common melanogenic target, tyrosinase. In all melanogenesis assay models we conducted, PAL-10 showed anti-melanogenic potencies superior to those of arbutin, strongly suggesting that PAL-10 may have a high potential as a novel de-pigmenting agent. Incidentally, we also assessed skin irritation potential of PAL-10 in 3D human epidermis model, Neoderm[®]-ED, according to OECD TG439, where PAL-10 did not affect the tissue viability up to 250 μ g/mL (>90 % of control, data not shown) while a positive control, 5 % sodium lauryl sulfate, decreased tissue viability to <10 %, ensuring the safety of topical application of PAL-10. Currently, formulation and clinical efficacy of PAL-10 are being explored and we hope that we can report the results in near future.

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