

## **Synthesis of 5-Aminolevulinic Acid–Based ALACELL Possessing Inhibitory Effect against *Cutibacterium acnes*: Whitening Effect and Protective Effect of UVB-Irradiated Damage Cells**

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### **Synopsis**

Five-aminolevulinic acid (5-ALA)-photodynamic therapy combined with infrared radiation is an effective and safe therapy for facial acne. Although there are various available agents for treating acne, therapies for resistant or severe strains have been limited. The aim of this study was to investigate the inhibitory efficacy of ALACELL synthesized by combining 5-ALA with Y-G-G-F-L peptide against *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli*, and *Yersinia enterocolitica*, as well as *Cutibacterium acnes*. Furthermore, other effects of ALACELL on human skin cells, melanin formation, intracellular tyrosinase activity, and Ultra Violet B (UVB)-irradiated cell death were measured by treatment of ALACELL *in vitro*. ALACELL particularly showed a growth inhibitory effect on *C. acnes* and no inhibitory effect on the four bacteria strains. ALACELL reduced melanin formation and intracellular tyrosinase activity by  $\alpha$ -melanin cell-stimulating hormone ( $\alpha$ -MSH) in B16F10 cells, with no cytotoxicity. ALACELL also improved cell viability in UVB-irradiated HaCaT cells. The results of the experiment show that ALACELL exhibits more efficacy than 5-ALA against antimicrobial activity, melanin formation, intracellular tyrosinase activity, and UVB-irradiated cell death. Therefore, ALACELL is recommended as a candidate for clinical application in the treatment of acne and skin aging and will be further investigated to study the mode of action and in actual situations.

### **INTRODUCTION**

All humans have a variety of micro-organisms on the skin that constitute the skin microbiome and that might play a role in skin immunity and skin barrier (1). Systemic imbalances in this skin have been related to dermatologic disease problems, such as acne and atopic dermatitis (2).

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*Cutibacterium acnes* is a Gram-positive and anaerobic bacterium detected in human skin that has been reported to represent about 90% of the micro-organisms found on a typical adult's face (3). However, *C. acnes* produces several enzymes that degrade skin constituents as well as chemotactic factors that excite keratinocytes and inflammatory cells to release pro-inflammatory cytokines and reactive oxygen species (ROS) (4,5). Many studies have also shown that some genes among the *C. acnes* genome service the virulence of bacteria and hence acne pathophysiology (6). Acne treatment using synthetic chemical drugs such as antibiotics and steroids can cause mild or severe side effects (7). Also, resistance of *C. acnes* against antimicrobials grew almost 40% between the 1980s and 2000s worldwide (8).

Cells responsible for skin pigmentation are committed to the regulation of melanogenesis (9). Reiterating exposure by ultraviolet (UV) irradiation leads to DNA damage in keratinocytes and induces production of the melanocyte-stimulating hormone ( $\alpha$ -MSH). Eventually, a UV-triggered pathway induces melanin synthesis and the transfer of melanosomes to keratinocytes (10). In East Asia, most women make efforts to reduce pigmentation and to improve skin lightening on their skin (11). The development of an effective whitening candidate to reduce toxicity and side effects from various materials is focused on with great interest in the cosmetic industry (12). However, active agents that exhibit inhibitory activity on melanogenesis have not been reported.

Therefore, a variety of research is focused on identifying the effectiveness of antibacterial or whitening properties that are preventative and therapeutically useful candidate materials to treat microbial infections or photoreaction. Five-aminolevulinic acid (5-ALA) is a precursor for the synthetic processing of tetrapyrrole compounds and is used variously in medicine and agriculture fields (13). *C. acnes* synthesizes and stores porphyrins, and the porphyrins are endogenous photosensitive sources that absorb light energy within range of a specific wavelength range, but the amount of porphyrins made by *C. acnes* is relatively low (14). Therefore, to heal skin lesions, one might topically apply exogenous compounds such as 5-ALA (15). ALA-photodynamic therapy (PDT) has the potential to provide the only way of making better acne treatment by selectively eliminating defective pilosebaceous units and killing *C. acnes* (16). In this process, energy is transferred from porphyrins to oxygen, and oxygen-reactive species such as singlet oxygen were generated, which effectively oxidize molecules to generate cytotoxicity in epithelial cells (17,18). Therefore, an agent that possesses more antimicrobial activity than 5-ALA in *C. acnes* was synthesized by combining 5-ALA with Y-G-G-F-L peptide (ALACELL) in this study. It investigated the antimicrobial activities of ALACELL for *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli*, and *Yersinia enterocolitica*, as well as *C. acnes*. This study also investigated the whitening effect of ALACELL with the measurement of melanin formation and intracellular tyrosinase activity. Furthermore, it measured the recovery effect of cell damage in UVB-irradiated HaCaT cells.

## MATERIALS AND METHODS

### CHEMICALS AND REAGENTS

5-ALA was purchased from Uniquemedicare Co., Ltd. (Gwangju-gwangyeoksi, South Korea). Phosphate-buffered saline (PBS), Trypsin-EDTA, fetal bovine serum (FBS), and Dulbecco's

modified Eagle's medium (DMEM) were supplied by Gibco BRL (Grand Island, NY).  $\alpha$ -melanocyte-stimulating hormone ( $\alpha$ -MSH), arbutin, dimethyl sulfoxide (DMSO), tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propylthiouracil (PTU), and phenylmethanesulfonyl fluoride (PMSF) were purchased from Sigma-Aldrich (St. Louis, MO).

#### SYNTHESIS AND PURIFICATION OF ALACELL

The peptides of the tyrosine-glycine-phenylalanine-leucine (Y-G-G-F-L) were produced according to a chemical synthesis method known in the relevant field of technology (19). ALACELL was synthesized by combining 5-ALA with Y-G-G-F-L peptides. ALACELL was synthesized by solid-phase peptide synthesis techniques, and then purified by RP-HPLC C18 column (Bondapark 5  $\mu$ m, 250  $\times$  4.6 mm, Waters, Ireland) in a gradient of acetonitrile in 0.1% trifluoroacetic acid at 215 nm. The peptide moiety Y-G-G-F-L was prepared with a standard solid-phase peptide synthesis method. A matrix-assisted laser desorption ionization time of flight mass spectroscopy (MALDI-TOF MS) assay (linear mode,  $\alpha$ -cyano-4-hydroxy-cinnamic acid matrix) was performed to ensure the synthetic quality of ALACELL (molecular weight and chemical structure). The MALDI-TOF MS assay (linear mode,  $\alpha$ -cyano-4-hydroxy-cinnamic acid matrix) was performed to ensure the synthetic quality of ALACELL (molecular weight and chemical structure). A MALDI-TOF MS instrument was used, along with an Axima curved-field reflectron (Shimadzu/Kratos, Manchester, United Kingdom) instrument, in which gauge pressure was set to  $8.0 \times 10^{-4}$  Pascal gauge pressure, linear mode, and 96-square well sample plate. MALDI-MS data were acquired using an AXIMA-CFR<sup>TM</sup> Plus-mass spectrometer (Shimadzu Biotech, Japan) in positive ion reflectron mode.

#### MICRO-ORGANISMS AND CULTURE

*C. acnes* was obtained from the Korean Culture Center of Microorganisms (KCCM, Seoul, South Korea). *C. acnes* was incubated in Gifu Anaerobic Medium (GAM) for 48 h at 37°C. *S. aureus*, *B. cereus*, *E. coli*, and *Y. enterocolitica* were obtained from the Chungcheongnam-Do Health and Environment Research Institute in Korea and were incubated for 48 h under aerobic conditions with brain heart infusion (BHI) broth (Difco, Lawrence, MA).

#### DETERMINATION OF ANTIMICROBIAL ACTIVITY

*C. acnes* was prepared by incubation in GAM broth for 48 h. *S. aureus*, *B. cereus*, *E. coli*, and *Y. enterocolitica* were prepared by incubation in BHI broth for 48 h. The concentration of microbial strains was adjusted to 0.5 OD<sub>620</sub> nm. A diluted microbial suspension (100  $\mu$ L) was inoculated into a 96-well microplate. The 50% inhibitory concentration (IC<sub>50</sub>) was determined in mM for the 5-ALA and ALACELL using a twofold serial dilution assay. Each compound was diluted by DMSO to a concentration of 1,000  $\mu$ M, and serial dilutions were conducted to make a range from 11.90 to 95.33 mM and from 2.33 to 18.70 mM, respectively. A diluted compound (100  $\mu$ L) was added to a 96-well microplate. A medium blank containing the selective broth and the compound solution was also made for the

controls. After incubation for 48 h, the absorbance was measured at 620 nm with a microplate reader (EZ read 400, Biochrom Ltd., Cambridge, UK). The results were transformed to a percentage of the controls. The IC<sub>50</sub> measure was graphically obtained from the dose–response curves.

#### CELL CULTURE AND CYTOTOXICITY

B16F10 melanoma cells and HaCaT keratinocyte cells that were purchased from the American Type Culture Collection (ATCC, Manassas, VA) were cultured in a 5% CO<sub>2</sub> incubator at 37°C. The cells were cultured in DMEM supplemented with 10% FBS and 0.01% antibiotic-antimycotic solution (Invitrogen, Grand Island, NY) in a 5% CO<sub>2</sub> incubator at 37°C.

The cytotoxic effect of 5-ALA and ALACELL was measured by the MTT assay (20). Briefly, cells were seeded uniformly at  $2.5 \times 10^4$  cells/well densities in 96-well microplates. After 24 h, the media were replaced with 100  $\mu$ L media containing final concentrations equivalent to 10, 20, and 40  $\mu$ M in 5-ALA and ALACELL. The MTT assay was performed after 48 h of incubation with 5-ALA and ALACELL. The cytotoxicity was quantified by measuring UV absorbance at 570 nm by a Tecan microplate reader (Mannedorf, Switzerland). The measured absorbance was standardized to the absorbance of nontreated control cells. All data represent the mean and standard deviation from at least three separate experiments and were compared using a student's *t*-test.

#### EFFECT OF ALACELL ON MELANIN FORMATION

The melanin formation was analyzed by a modification of the method described by a previous study (21). B16F10 melanoma cells were seeded at a density of  $3 \times 10^5$  cells/well in 96-well microplates and cultivated by the method described earlier. To induce hyperproduction of melanin, the cells were treated with  $\alpha$ -MSH of 100 nM. Then, the concentrations of 10, 20, and 40  $\mu$ M of 5-ALA and ALACELL were added to the medium and further incubated for 48 h. To remove melanin excreted from the cells, the medium was removed, and the cells were washed twice with PBS, and then harvested by trypsin treatment. The harvested cells were pelleted, and the cell membrane was dissolved in Triton X-100 (Sigma-Adrich, St. Louis, MO). The purified melanin was dissolved in 2 M NaOH for 30 min at 100°C. The absorbance was measured at 405 nm. The melanin content was compared with untreated control cells.

#### EFFECT OF ALACELL ON INTRACELLULAR TYROSINASE ACTIVITY

Intracellular tyrosinase activity can rapidly oxidize *L*-tyrosine to *L*-3,4-dihydroxyphenylalanine (*L*-DOPA) and further convert it to dopaquinone, and then the activity of tyrosinase determines the amount of brown dopaquinone (22,23). In this study, B16F10 melanoma cells were seeded at a density of  $3 \times 10^5$  cells/well in 96-well microplates, and the culture medium was discarded after 24 h. Five-ALA and ALACELL were treated with 10, 20, and 40  $\mu$ M. After 48 h, the cells were washed twice with PBS. B16F10 melanoma cells were lysed in 1% Triton X-100, 20 mM sodium phosphate (pH 6.8), and 1 mM

PMSE, and centrifuged at 14,000 rpm for 15 min. The Bradford assay with bovine serum albumin as the protein standard was conducted to measure the protein content of the supernatant. Intracellular tyrosinase activity was measured in a reaction mixture (1 mL) containing 50 mM phosphate buffer (pH 6.8), 2 mM L-DOPA, and 300  $\mu$ g supernatant proteins. After incubation at 37°C for 15 min, the absorbance was measured at 475 nm by a microplate reader. Tyrosinase activity (%) =  $[(A-B)/C] \times 100\%$ ; A: sample absorbance volume, B: blank absorbance volume, and C: control absorbance volume.

#### EFFECT OF ALACELL ON UVB-IRRADIATED HaCaT CELLS

UVB irradiation was performed by UVM-225D Mineralight UV display lamps (UVP, Phoenix, AZ) generating UVB light in the range of 290–320 nm with a maximum emission wavelength of 302 nm. UV doses were measured using a HD2102-2 UV meter (Delta OHM, Padova, Italy). HaCaT keratinocyte cells were seeded on six-well plates at  $7 \times 10^4$  cells/well and incubated in a 5% CO<sub>2</sub> incubator at 37°C. Five-ALA and ALACELL were treated in cultured cells at concentrations of 20, 40, 80, and 100  $\mu$ M, and after 1 h, the cells were irradiated with UVB at 40, 80, and 120 mJ/cm<sup>2</sup>. To prevent UV quenching, before irradiation, the cell culture medium was replaced by the same volume of PBS after two washing steps with PBS. After UVB irradiation, cells were fed with fresh growth medium and incubated. After 24 h, the cell viability was measured by the MTT assay described earlier.

#### STATISTICAL ANALYSIS

Results are expressed as means  $\pm$  standard deviation. Data were analyzed using analysis of variance and Duncan's multiple range tests. Significance was indicated at \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$ .

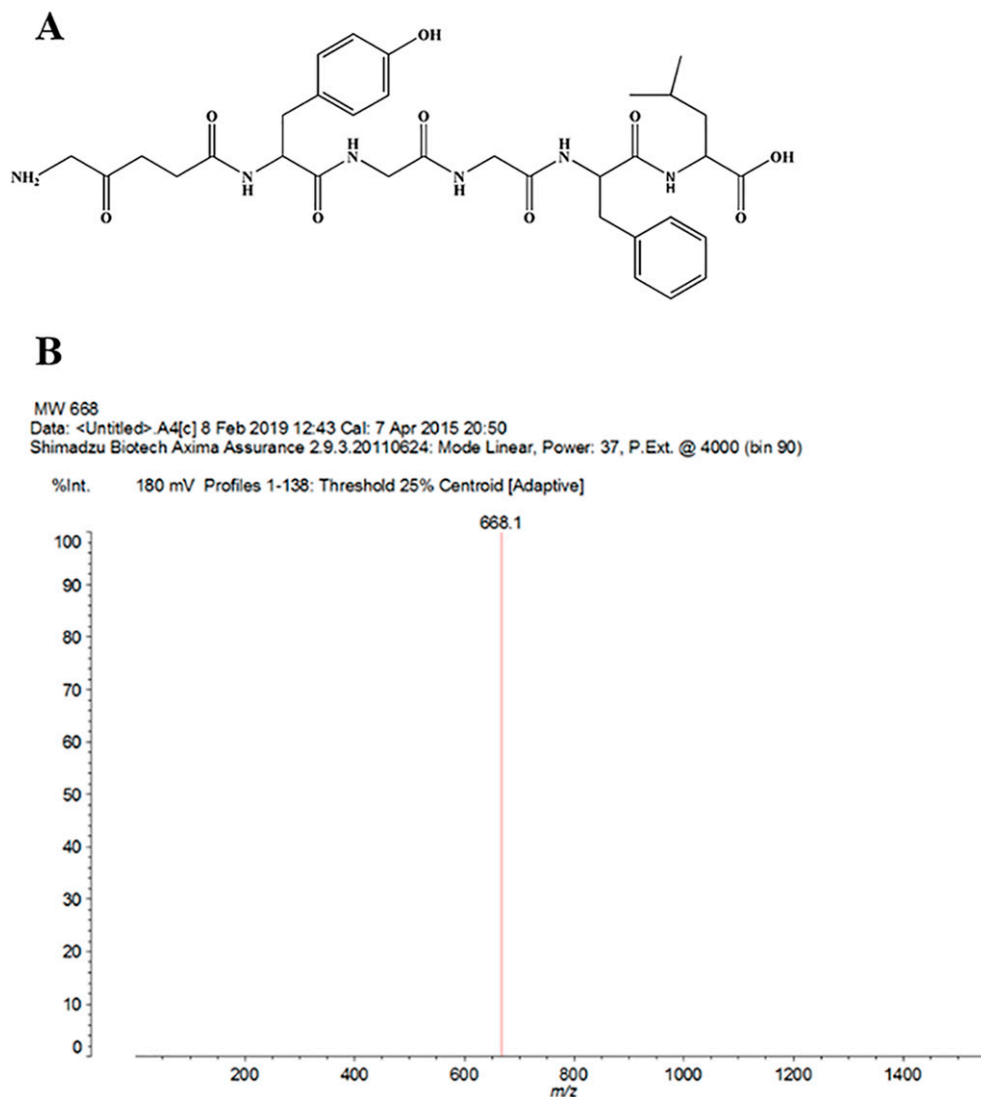
## RESULTS

#### CONFIRMATION OF ALACELL STRUCTURE BY MALDI-TOF MS

ALACELL was synthesized by combining the phytochemical agent 5-ALA with Y-G-G-F-L peptide to support physicochemical activity. MALDI-TOF mass spectrometry was performed to identify the molecular weight and chemical structure of ALACELL (Figure 1A). The chemical formula of ALACELL is C<sub>33</sub>H<sub>44</sub>N<sub>6</sub>O<sub>9</sub>, and its molecular weight is 668.32 (Figure 1B).

#### GROWTH INHIBITORY EFFECT OF ALACELL AGAINST *C. ACNES*

To investigate the antimicrobial activities of 5-ALA and ALACELL, we determined the minimum concentration that produces 50% inhibition of bacterial growth (IC<sub>50</sub>) by the broth dilution assay. We tested different concentrations for each sample against *C. acnes*, *S. aureus*, *B. cereus*, *E. coli*, and *Y. enterocolitica*. 5-ALA and ALACELL against *C. acnes* blocked growth dose dependently, respectively (Table I). The IC<sub>50</sub> of 5-ALA and ALACELL against



**Figure 1.** Chemical structure of ALACELL (A) and analysis by MALDI-TOF mass spectrometry (B). ALACELL was synthesized by combining 5-ALA with Y-G-G-F-L peptide. After purifying by reverse-phase HPLC of ALACELL, a MALDI-TOF mass spectrometry was performed and MALDI-MS data were analyzed by using AximaCFR™ Plus-mass spectrometer in positive ion reflectron mode.

*C. acnes* were identified as 18.01 and 6.12 mM, respectively (Table I). The inhibitory effect of ALACELL against *C. acnes* was higher than that of 5-ALA. However, antimicrobial activity against *S. aureus*, *B. cereus*, *E. coli*, and *Y. enterocolitica* did not appear (data not shown).

#### ALACELL DECREASED MELANIN FORMATION WITHOUT CYTOTOXICITY IN B16F10 CELLS

The effect of 5-ALA and ALACELL on B16F10 melanoma cell proliferation showed that both compounds did not have significant cytotoxicity against the concentration test

Table I  
The IC<sub>50</sub> of 5-ALA and ALACELL in *C. acnes*

| Concentration         | 5-ALA       | ALACELL     |
|-----------------------|-------------|-------------|
| IC <sub>50</sub> (mM) | 18.01 ± 0.3 | 6.17 ± .4.0 |

(Figure 2A and B). To study the effect of ALACELL on melanin formation, the melanin formation of 10, 20, and 40  $\mu\text{M}$  on ALACELL-treated B16F10 melanoma cells was quantified. Treatment of  $\alpha$ -MSH (100 nM) induced a significantly increase of melanin formation (137.2%,  $p < 0.001$ ; Figure 2C). Arbutin (40  $\mu\text{M}$ ) and PTU (75  $\mu\text{M}$ ) significantly reduced melanin formation in cells induced by  $\alpha$ -MSH (Figure 2C;  $p < 0.01$  and  $p < 0.001$ , respectively). Five-ALA and ALACELL decreased melanin formation in  $\alpha$ -MSH-induced cells, dose dependently (Figure 2C). Among the two compounds, ALACELL decreased more melanin formation at 10, 20, and 40  $\mu\text{M}$  than 5-ALA, respectively. These results indicate that ALACELL exhibits antimelanogenic efficacy in B16F10 melanoma cells without cytotoxic effect.

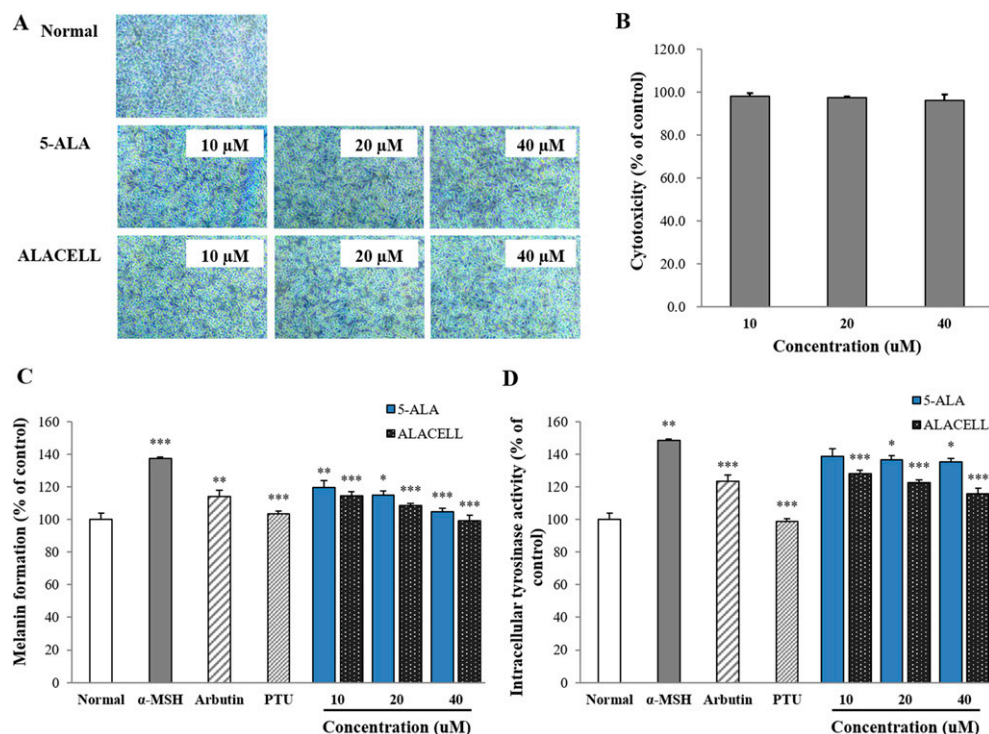


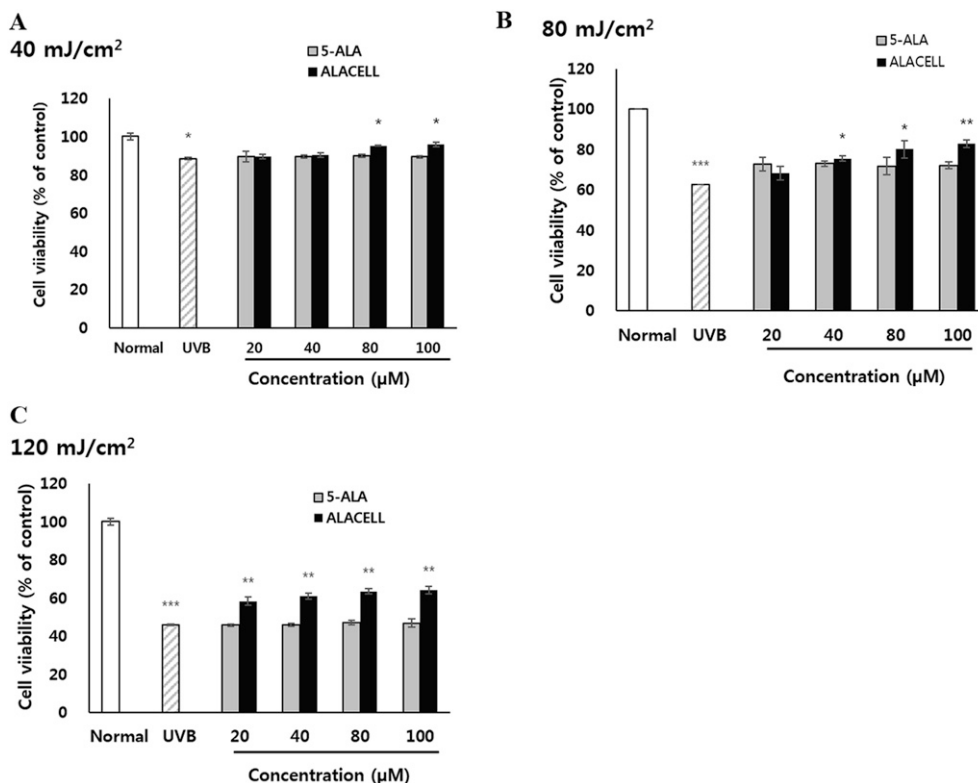
Figure 2. The cytotoxicity by treatment of ALACELL (A and B), inhibitory effect of ALACELL on melanin formation (C), and inhibitory effect of ALACELL on tyrosinase activity in B16F10 cells (D). B16F10 cells were treated with concentrations of 10, 20, and 40  $\mu\text{M}$ . After incubation of 48 h, the cells were photographed under a microscope of  $2.5 \times 10^4$  magnifications, and cytotoxicity was measured by the MTT assay. Data are displayed with mean  $\pm$  standard deviation ( $n = 3$ ). Significance was indicated at  $*p < 0.05$ ,  $**p < 0.01$ , and  $***p < 0.001$ .

## ALACELL INHIBITS TYROSINASE ACTIVITY WITHOUT CYTOTOXICITY IN B16F10 CELLS

Five-ALA and ALACELL (10, 20, and 40  $\mu\text{M}$ ) significantly inhibited intracellular tyrosinase activity as compared with B16F10 melanoma cells treated with  $\alpha$ -MSH. In addition, the ALACELL and 5-ALA were shown to reduce tyrosinase activity by 115.6% and 135.4% at 40  $\mu\text{M}$ , respectively (Figure 2D,  $p < 0.001$ ).

## ALACELL IMPROVES CELL VIABILITY IN UVB-IRRADIATED HaCaT CELLS

To evaluate whether ALACELL protected HaCaT keratinocyte cells against UVB-induced cell death, we detected the viability of HaCaT cells after being exposed to UVB (40, 80, and 120  $\text{mJ}/\text{cm}^2$ ) and incubation for 24 h, with and without ALACELL treatment at concentrations of 20, 40, 80, and 100  $\mu\text{M}$ . As shown in Figure 3, the viability of HaCaT cells was decreased significantly to 88.4% after being exposed to UVB (40  $\text{mJ}/\text{cm}^2$ ) and for 24 h. 5-ALA did not display recovered viability in UVB-irradiated (40, 80, and 120  $\text{mJ}/\text{cm}^2$ )



**Figure 3.** Protective effect of ALACELL by 40  $\text{mJ}/\text{cm}^2$  (A), 80  $\text{mJ}/\text{cm}^2$  (B), and 120  $\text{mJ}/\text{cm}^2$  (C) irradiation on UVB-irradiated HaCaT cells. The HaCaT cells were seeded on six-well plates at  $7 \times 10^4$  cells/well and treated with 40, 80, and 120  $\text{mJ}/\text{cm}^2$  of UVB for 1 h. The cells were treated with 20, 40, 80, and 100  $\mu\text{M}$  of ALACELL for 12 h, and then followed by UVB treatment. After 24 h, cell viability was measured by the MTT assay. Data are displayed with mean  $\pm$  standard deviation ( $n = 3$ ). Significance was indicated at \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$ .



cells at four concentrations (Figure 3A). However, cells treated with ALACELL for 24 hr at concentrations of 80 and 100  $\mu\text{M}$  before the UV irradiation were significantly protected against the loss of viability (Figure 3A and  $p < 0.05$ ). Also, the survival of HaCaT cells exposed to UVB (80  $\text{mJ}/\text{cm}^2$ ) for 24 h decreased significantly to 62.56% ( $p < 0.001$ ), and ALACELL concentrations of 80 and 100  $\mu\text{M}$  for 24 h significantly increased cell viability (Figure 3B,  $p < 0.05$  and  $p < 0.01$ , respectively). The UVB-irradiated (120  $\text{mJ}/\text{cm}^2$ ) HaCaT cells showed cell viability of 45.78% (Figure 3C and  $p < 0.001$ ), but ALACELL treated with four concentrations significantly recovered cell viability (Figure 3C and  $p < 0.01$  for four concentrations).

## DISCUSSION

Acne is a type of chronic inflammatory disease that general appears as pustules (pimples) and nodules on human skin (24). Furthermore, acne can generate psychological and social issues, and have a grave effect on the quality of human life (15). Therefore, many researchers are focused on detecting safe and effective ways to treat acne.

A variety of treatments are available for acne, including antibacterial agents, natural compounds, and hormone therapy. However, these treatments have limitations in the clinic, where, for example, retinoids have several side effects and antibiotics may cause resistance (25).

PDT has played an important role in dermatological treatment and offers alternatives to people who perform topical treatments. Treating 5-ALA-PDT, 5-ALA is taken in by epithelial cells and synthesized into protoporphyrin IX through the biosynthetic pathway, and then photoactivated porphyrin is formed from the making of singlet oxygen and other potent oxidizers that produce antimicrobial and anti-inflammatory efficacy (16). In this study, ALACELL showed growth inhibitory effect against *C. acnes*, but there was no inhibitory effect against other strains (*S. aureus*, *B. cereus*, *E. coli*, and *Y. enterocolitica*). In addition, the inhibitory effect of ALACELL on *C. acnes* was higher than that of 5-ALA. Skin aging is a biochemical process caused by many individual factors, such as UV light exposure (26). Various factors cause skin aging, including wrinkles and pigmentation (27). In this study, to look for other effects of ALACELL on human skin as well as its inhibitory effect on *C. acnes*, melanin formation and tyrosinase activity from the treatment of ALACELL were measured in B16F10 melanoma cells. As a result, ALACELL significantly decreased the  $\alpha$ -MSH-induced melanin formation and tyrosinase activity in B16F10 cells dose dependently, and ALACELL was higher than 5-ALA. Therefore, this study found that 5-ALA-based synthesized ALACELL efficiently showed whitening effects, resulting in the decrease of  $\alpha$ -MSH-induced melanin formation and tyrosinase activity in B16F10 cells.

Continuous UVB irradiation permeates human skin and produces intracellular ROS, resulting in various skin changes, including rough skin, dryness, and wrinkle formation (28). Consumers are increasingly looking to various ingredients to improve the appearance of their skin and to delay the effects of aging. This study evaluated the protective effects of ALACELL on UVB-irradiated cell death by irradiation of 40, 80, and 120  $\text{mJ}/\text{cm}^2$ . ALACELL concentration of 80 and 100  $\mu\text{M}$  significantly increased cell viability at irradiations of 40, 80, and 120  $\text{mJ}/\text{cm}^2$  for 24 h. Its protective efficacy was higher than that of 5-ALA. From these results, it is concluded that the 5-ALA-based synthesized ALACELL may be a photoprotection candidate for skin care.

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