Master of Science Degree

# L-765,314 inhibits melanogenesis by controlling PKC activity

# L-765,314의 멜라닌 형성 억제 기능에 대한 연구

The Graduate School

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# L-765,314 inhibits melanogenesis by controlling PKC activity

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# Abstract

While melanin production is a beneficial process that protect skin tissue from a variety external hazard, dysregulation of it causes skin hyperpigmentary disorders. Despite numerous side effects of existing therapeutic drugs, new drugs have not been proposed. In this study, we conducted high-throughput melanin content assay based screening and identified L-765,314 ((2S)-4-(4-amino-6,7-dimethoxy-2-quinazolinyl)-2-[[(1,1-dimethylethyl)amino]carbonyl]-1-piperazinecarboxylic acid) reduced melanin production.

We found that L-765,314 did not influence cell viability, the mRNA and protein expression levels of the genes associated with melanin formation, including Microphthalmia-associated transcriptian factor (MITF), Tyrp1, DCT, and Tyrosinase. However, L-765,314 downregulated tyrosinase activity and melanin production. This seems to be mediated by reduced PKC activity, as evidenced by the decreased level of PKC substrate phosphorylation in Mel-ab and human melanocytes. These results

suggest that L-765,314 could be a potential therapeutic candidate for skin hyperpigmentary disorders and discovery of selective inhibitors targeting PKC might be a promising strategy for the development of depigmenting agents to treat hyperpigmentary disorders.

Key words: melanocytes / L-765,314 / melanogenesis / ADRA1b antagonist / PKC / Tyrosinase activity

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# Introduction

## Skin hyperpigmentary disorders

Skin tissues play a major role in defense against a wide range of environmental threats. In their position at the forefront of our body's defenses, skin tissues are directly exposed to ultraviolet radiation (UVR); repeated UVR exposure increases the risk of carcinogenesis via the induction of DNA damage and mutation [1]. Most diseases caused by UVR are local hyperpigmentation disorders, such as freckles, black spots, and mellitus, of which main causative is an increased melanin production due to ultraviolet exposure.

In the cosmetic purpose, whitening was one of the highly interesting areas. Therefore, many researchers have paid attention to the treatment of skin diseases associated with melanin hyperplasia and inhibition [2-3]. Placenta extract, arbutin, Kojic acid, polyphenols, and flavonols, well-known inhibitors of the production and activity of tyrosinase, have been used to inhibit the production of melanin [4]. In addition,

coenzyme Q10 and the green tea extract, which removes active oxygen, and hydroquinone and retinol (vitamin A derivative) were used to remove created melanin. However, these drugs can cause side effects such as skin irritation, inflammation, erythema, itching, skin discoloration, and high-doses hydroquinone can cause gene mutation and carcinogenicity. It is necessary to search for advanced drugs that have no side effects [5]. In order to develop an effective and safe therapeutic agent, we screened a drug that inhibits melanin production in mouse melanoma cells called B16-F10, applied the active molecules to normal mouse melanocytes named Mel-ab cells and investigated as to which of the mechanism is involved in the melanin production.

**Melanin formation**

In animals, melanin is synthesized at specialized lysosome-related organelles, termed melanosomes, in melanocytes and is transported to and accumulates in the

keratinocytes. Melanin is a produced in various tissues such as skin, eyes, and hair of various animals and serves to determine the skin and hair color, shield from UV rays and for maintenance of the skin temperature [6]. The process of melanin synthesis on the melanocytes is called melanogenesis, and melanocytes produce eumelanin and pheomelanin [7]. Eumelanin is brown or black, and pheomelanin is a reddish yellow that acts to create spots on the stain, these two melanin mixes produce different skin colors, eyes and hair color [8-9]. The process of melanogenesis begins with the exposure of skin tissues to UV light. The melanosome in the melanocytes basal layer activates tyrosinase. Tyrosinase is the first and rate-limiting step of melanogenesis. Tyrosinase oxidizes L-tyrosine to form L-DOPA and then converts to dopaquinone (DQ). Finally, dopaquinone forms pheomelanins. In addition, eumelanin is formed when TRP1 (= TYRP) and TRP2 (= DCT) enzymes, which are known to work with tyrosinase in melanogenesis, act on dopaquinone [7][10]. This melanin is transferred to the keratinocytes through the dendrites of melanocytes [7]. In addition to melanocytes and keratinocytes, there are many paracrine factors that involved in

melanogenesis such as α-melanocyte stimulating hormone (α-MSH), the melanocortin 1 receptor (MC1R) and endothelin-1 (EDN-1), interleukin-2 (IL-2), basic fibroblast growth factor (bFGF), stem cell factors (SCF), adrenocorticotropic hormone (ACTH), prostaglandins, leukotrienes, thymidine dinucleotide and histamine [9].

**Biological action of L-765,314**

Adrenergic receptors are divided into subtypes of α1, α2, β1, β2, and β3 depending on the affinity of epinephrine, norepinephrine and isoproterenol, which are catecholamines. α1 adrenergic receptors are divided into α1A, α1B and α1D subtypes depending on the reaction of the α-receptor.

L-765,314 ((2S)-4-(4-amino-6,7-dimethoxy-2-quinazolinyl)-2-[[(1,1-dimethylethyl) amino] carbonyl]-1-piperazinecarboxylic acid) is a selective antagonist of ADRA1b (adrenergic receptor subtype α1B) [11-12]. ADRA1b is one of the G protein classes that control the Gq signal, acting as G protein-coupled receptors (GPCRs) and

dividing into Gα and Gβ-Gγ complexes, which function as secondary messengers within the cell.

**PKC signaling pathway**

When the hormone binds to the G-protein coupled receptors (GPCR) localized in the plasma membrane phospholipid bilayer, phospholipase C (PLC) is activated through the G-protein and PLC degrades phosphatidyl inositol-4,5-biphosphate (PIP2), a part of the cell membrane phospholipid, to produce a second messenger called inositol triphosphate (IP3) and diacylglycerol (DAG). The function of IP3 is to increase the intracellular calcium ion ($Ca2^{+}$) concentration by releasing the calcium ion stored in the endoplasmic reticulum (ER) to the cytoplasm. DAG activate the protein kinase C (PKC) in the vicinity of the cell membrane. There are more than 10 isoforms in PKC, and the form most commonly involved in melanogenesis is known as β-form of PKC [13].

**Previous research progress**

The function of Gs coupled GPCR and PKA signaling pathway in melanin production have been intensively studied [14-16]. ERK and AKT have shown to control melanogenesis by regulating Microphthalmia-associated transcriptian factor (MITF) stability [17]. However, the relationship between ADRA1b in melanogenesis has not been addressed yet. L-765,314 has been best characterized in the nervous and vascular system, but its effect on skin tissues or melanin synthesis has not been reported [18-22]. Previous studies on tyrosinase mainly focused on mushroom tyrosinase. However, the mushroom is not a significant result for animals because it belongs to a different system than mouse or human [23]. Therefore, we investigated the role of tyrosinase in melanogenesis using mouse and human cells [24].

**Significance of this study**

The cosmeceutical market, such as functional cosmetics and therapeutic cosmetics for the treatment of various skin diseases, has maintained a steady growth rate. Therefore, through this research, we hope to contribute for the development of cosmetics and therapeutic agents.

# Materials and methods

**1. Materials**

The LOPAC library (Sigma-Aldrich, St Louis, MO, USA) was used for screening for melanin production in B16-F10 cells. L-765,314 ((2S)-4-(4-amino-6,7-dimethoxy-2-quinazolinyl)-2-[[(1,1-dimethylethyl) amino] carbonyl]-1-piperazinecarboxylic acid, L3040) and TPA (P8139) were purchased from Sigma-Aldrich (St Louis, MO, USA). Forskolin (FSK, No. 1099) and cirazoline hydrochloride (CRZ, No. 0888) were purchased from Tocris Bioscience (Bristol, UK) and phenylephrine hydrochloride (PE, P0398) purchased from Tokyo Chemical Industry (Tokyo, Japan).

**2. Screening for the inhibition of melanin formation (HTS)**

B16-F10 cells are cultured on a 96-well plate with $1 \times 10^4$ cells per well in phenol red free DMEM with 10% FBS. After 24 hours, 500 nM of $\alpha$-MSH and 0.1 μM, 1 μM, and 10 μM concentration of individual compounds from 1280 of the LOPAC library

(Sigmal-Aldrich, St Louis, MO, USA) compounds were treated. Three days after the treatment, the medium was removed and the absorbance at 405 nm was measured to confirm the inhibition of melanin formation in the B16-F10 cells. In order to confirm the toxicity of the compounds, MTT based cell viability assay was carried out.

**3. Cell culture**

(1) B16-F10 cells and HEK-293T cells were cultured in DMEM (Corning, 10-013-CVR) medium containing 10% FBS (Corning, 35-015-CV) and 1% antibiotics (Hyclone, SV30010). For experiments using B16-F10 cells, 10% FBS (Corning, 35-015-CV), L-glutamine, Sodium pyruvate and 1% antibiotics (Hyclone, SV30010) were added to phenol red free media (Gibco, 31053-028). B16-F10 cells were subcultured every other days.

(2) Mel-ab melanocytes were maintained in DMEM (Corning, 10-013-CVR) with 10% FBS (Corning, 35-015-CV), 1% antibiotics (Hyclone, SV30010), 1 nM Cholera Toxin

(Cayman chemical, Cholera Toxin from Vibrio cholera, 23217) and 100 nM TPA medium. Culture media was replaced with fresh media every other days. For the melanogenesis assay, DMEM (Corning, 10-013-CVR) medium containing 10% FBS (Corning, 35-015-CV) and 1% antibiotics (Hyclone, SV30010) was used.

(3) For human melanocytes, 1% antibiotics (Hyclone, SV30010) were added to 254 medium (Thermo fisher scientific, M254500) and $6x10^5$ cells in a 75T flask about 10 days.

**4. Measurement of melanin contents**

Mel-ab cells were plated at $6\times10^5$ cells / well in a 6-well plate and treated with L-765,314 at concentrations ranging from 0.1 μM to 20 μM, and then photographed with a microscope every 24 hours and melanin content was measured 96 hours after initial treatment. For melanin content assay, cells were washed with PBS then 300 μl of 1N NaOH was applied for lysis, followed by boiling at 100℃ for 30 minutes. After

cooling at room temperature, lysates were centrifuged at 13000 RPM for 5 minutes. After transferring the supernatant to a new microtube, approximately 20 μl is used to quantitate the amount of BCA, and the melanin content was measured with remaining lysates by reading optical density at 405 nm

**5. Cell viability assay (MTT assay)**

$1.5\times10^5$ cells were placed per well in a 24-well plate and 20 μl of MTT (5 μg/μl) solution as added 1 hour before treatment. After incubation at 37℃ for 1 hour with the MTT reagent kit (Duchefa-biochemie, Haarlem, Netherlands), the medium was removed when purple crystals showing up, and 150 μl of DMSO is used to measure the viability by measuring with triplicate at 565 nm.

**6. Measurement of tyrosinase activity**

$6x10^5$ Mel-ab cells/well were plated in a 6-well plate, treated with the drug as

indicated in the figure legends, and washed with cold PBS. Then, 300 μl of tyrosine lysis buffer was used and followed by 8-10 times repeating of freezing/thawing. When the cells are completely destroyed, centrifuged at 13000 RPM, 4℃, 10 min, and protein amounts were quantified with BCA reagent assay. The lysates were aliquoted to give a total volume of 90 μl (triplicate) at a concentration of 100μg/90ul. Before measurement, 10 μl of L-Dopa (Sigma, D9628, 10 mM) was added and tyrosinase activity was measured by reading optical density at 475 nm every 10 minutes while incubating at 37℃. M-tyrosine is used as a positive control and the sample buffer is used as a negative control.

**7. Western blot**

For Western blot, the cells are washed once with PBS, and then 120 μl of 1% SDS buffer is added per well in 12-well plates. After harvesting the cells, 30 μl of 5x sample buffer was added and incubated at 98℃ for 10 minutes followed by centrifuged at room

temperature. Protein samples were separated by SDS-PAGE and transferred on to nitrocellulose (NC) membrane at 0.2A per cassette for 80 minutes. After blocking for 1 hour with 3% BSA, the primary antibody was incubated in a cold room for overnight. The next day, membrane was incubated with HRP conjugated secondary antibody (Rabbit, Mouse, Goat 1: 5000 each). The primary antibodies for HSP90 (1: 1000, SC-7947, Santa Cruz biotech), Tyrosinase (M-19) (1: 1000, SC-7834), TRP1 (G-17) (1:1000, SC-10443), TRP2 (D-18) (1:1000, SC-10451) were purchased from Santa Cruz Biotech. Antibody for Microphthalmia-associated transcriptian factor (MITF, Ab-1 (C5)) was purchased from Neomarkers Inc. (MS-771-p1) and used as 1: 500 dilution.

**8. RNA expression test (qRT-PCR)**

Mel-ab, B16-F10, keratinocytes, and human dermal fibroblast cells were grown in 24 well plate and RNA was obtained using the farvogen RNA prep kit (FABRK001-2). cDNA was synthesized using 700 ng RNA and primer mix, 5x RT buffer, and RT

enzyme mix (ReverTra Ace® qPCR RT Kit (Toyobo, Osaka, Japan)). Each cDNA was subjected to qRT-PCR reaction using Lightcycler 480 (Roche Applied Science, IN, USA) / THUNDERBIRD™ SYBR® qPCR Mix (Toyobo). All the qRT-PCR components are mixed together and are taken through series of cyclic reactions. pre-incubation at 94℃ for 3 minutes, denaturation at 94℃ for 15 seconds, annealing at 60℃ for 30 seconds, elongation at 72℃ for 15 seconds. qRT-PCR cycle was repeated 45 times.

**Table 1. Primer list for qRT-PCR.**

| Gene | Forward primer | Reverse primer |
|---|---|---|
| **L32** | 5′-AGATCCTGATGCCCAACATC-3′ | 5′-CAGCTCCTTGACATTGTGGA-3′` |
| **mADRA1a** | 5′-GCTCCGTATCCACCGTAAAA-3′ | 5′-TCTCTCGGGAAAACTTGAGC-3′ |
| **mADRA1b** | 5′-GAGGCTGCGCTTACACCTAC-3′ | 5′-CTGCCACTGTCATCCAGAGA-3′ |
| **mADRA1d** | 5′-TCCGTAAGGCTGCTCAAGTT-3′ | 5′-GGACGAAGAAAAAGGGGAAC-3′ |
| **hADRA1a** | 5′-ACATTTCCAAGGCCATTCTG-3′ | 5′-CAGGCTACGGAGAGGATCAC 3′ |
| **hADRA1b** | 5′-TCTCCAGGGAAAAGAAAGCA-3′ | 5′-GCGGTAGAGCGATGAAGAAG-3′ |
| **hADRA1d** | 5′-CTCAGAGGTGGAGGCTGTGT-3′ | 5′-TATCGGTCTCCCGTAGGTTG-3′ |
| **MITF** | 5′-TGAAGCAAGAGCATTGGCTA-3′ | 5′-TCCACAGAGGCCTTGAGAAT-3′ |

| | | |
|---|---|---|
| **Tyrosinase** | 5′-TTATGCGATGGAACACCTGA-3′ | 5′-ACTGGCAAATCCTTCCAGTG-3′ |
| **Tyrp1** | 5′-TCACTGATGCGGTCTTTGAC-3′ | 5′-CTGACCTGGCCATTGAACTT-3′ |
| **DCT** | 5′-TCCTAACCGCAGAGCAACTT-3′ | 5′-TCTCCATTAAGGGCGCATAG-3′ |

**9. Measurement of promoter activity**

HEK-293T cells were plated in a 24 well plate at $1.5\times10^5$ cells per well. Each well was transfected with 150 ng MITF-Luc reporter plasmid or 150 ng Tyrosinase-Luc reporter plasmid and 50 ng RSV-β-gal plasmid as indicated in the figure legends. PEI (1 mg / ml) was added at a rate of 3 μl per 1 μg of DNA. 10 μM of L-765,314 and/or 10 μM of FSK were treated 24 hours after transfection. After 6 hours, the medium was removed and harvested with 150 μl of Luciferase lysis buffer (1 × gly-gly, 1% Trypton X, 100 mM ATP, 1M DTT) and transferred to a 96-well plate. Harvested samples are frozen in -80℃ deep freezer. After 30 minutes, lysates were centrifuged at 1000 RPM for 5 minutes and 25 μl of each well was transferred to a white 96-well plate. 25 μl of assay mix and 50 μl of Luciferin were added and measured by luminescence in a microplate

reader (BioTek, Winooski, Vermont, USA). For β-gal assay, 25 μl of lysates were mixed with 25 μl of β-gal Assay mix and incubated at room temperature for about 10 minutes till the colors turns yellow. Subsequently, the absorbance at 420 nm was measured with a microplate reader (BioTek, Winooski, Vermont, USA).

# Result

**Effect of L-765,314 on melanin contents in B16-F10 cells**

To investigate the inhibitory effects of the L-765,314 (Fig. 2) on melanogenesis, we treated 0.1, 1 and 10 μM of L-765,314 to B16-F10 mouse melanoma in 12 well plate for 72 hours. L-765,314 significantly decreased the melanin production (Fig. 1). As a positive control, we treated FSK to B16-F10 and verified that melanin production was stimulated by FSK.

**Effect of L-765,314 on melanin contents in Mel-ab cells**

To examine the effects of L-765,314 on melanogenesis in normal melanocytes, we treated 0.1-20 uM of L-765,314 on Mel-Ab mouse melanocytes. Melanin content was assessed by microscopic observation (Fig. 3) and melanin content assay (Fig. 4). Melanin accumulation was decreased by 8% at 2 μM, 9% at 2 μM, 19% at 5 μM, 28% at 10 μM, and 30% at 20uM of L-765,314 treatments (Fig. 4). In contrast, the

accumulation of melanin was increased by 43% in Mel-Ab cells treated with FSK. Moreover, pretreatment of L-765,314 on Mel-ab cells was able to inhibit the melanin synthesis stimulated by FSK (Fig. 5, 6). In order to test the toxicity of L-765,314, Mel-ab cells were treated with 0.1-20 uM of L-765,314 for 96 hours and MTT assay was conducted. We found that up to 20 uM of L-765,314 did not alter the cell viability (Fig. 7). To examine the expression of $\alpha$1 adrenergic receptor in epidermal skin resident cells; melanocytes and keratinocytes, we conducted qRT-PCR analysis. All three subtypes of $\alpha$1 ($\alpha$1A, $\alpha$1B, $\alpha$1D) receptors were expressed in Mel-ab cells, B16-F10 cells and human keratinocytes (Fig. 8).

**Effect of Agonists of Adrenergic Receptor 1 on Mel-ab cells**

To determine L-765,314's effect on melanogenesis is mediated through $\alpha$1B adrenoreceptors signaling, we treated $\alpha$1 adrenergic receptor agonists; cirazoline (CRZ) and phenylephrine (PE) in Mel-ab cells and tested whether $\alpha$1 adrenergic

receptor agonists have stimulating effect on melanogenesis. However, 5 uM of CRZ nor PE, at which concentration stimulates $\alpha$1 adrenergic receptor signaling, appeared to have significant effect on the melanin contents (Fig. 9), and higher dose of CRZ and PE (10~15 μM) did not affect the production of melanin either (Fig. 10). Therefore, we concluded that adrenergic receptor 1 signaling was not directly involved in the regulation of melanin production.

**Investigation on gene expression of sample with L-765,314 treatment in Mel-ab cells**

Protein samples were prepared from Mel-ab cells treated with 10 μM of L-765,314 for 4, 24, 48, 72, and 96 hours and the expression of MITF, Tyrosinase, TRP1, and TRP2 genes, known to be involved in the formation of melanin, was examined by western blot. None of aforementioned protein expressions were changed (Fig. 11). Because we couldn't see differences at the protein level, we examined mRNA expression of MITF,

Tyrosinase, TRP1, and TRP2. Similar to protein expression levels, mRNA levels of all four genes were not decreased by 10 μM of L-765,314 (Fig. 12). Corresponding to mRNA results, neither MITF nor tyrosinase promoter activity was suppressed by L-765,314 treatment (Fig. 13). Contrary to comparable tyrosinase expression at protein or RNA, tyrosinase activity was significantly decreased by 10 μM of L-765,314 (Fig. 14). Taken together these results showed that L-765,314 did not inhibit the expression of melanin-associated genes, it decreased melanin production by suppressing tyrosinase activity.

**Changes in PKC activity in Mel-ab cells with L-765,314 treatment**

Tyrosinase activity is known to be controlled by PKC signaling via direct induction of tyrosinase phosphorylation [25]. To investigate the effect of L-765,314 on PKC activity, protein samples were prepared from Mel-ab cells treated with 10 μM of L-765,314 for 96 hours and PKC activity was examined by western blot with phosphor-

PKC substrate antibody. Whereas 2-O-Tetradecanoylphorbol-13-acetate (TPA), a PKC activator, treatment increased PKC activity, L-765,314 pretreatment significantly decreased PKC activity in Mel-Ab cells (Fig. 15). Moreover, tyrosinase activity and melanin production stimulated by TPA was also attenuated by L-765,314 treatment (Fig. 16, 17).

**Human melanocytes was treated with L-765,314**

Finally, to investigate if the anti-melanogenic activity of L-765,314 seen in Mel-ab cells is applicable to human skin, we treated L-765,314 in normal human melanocyte (NHM) cells. Similar to Mel-Ab cells, melanin contents of NHM cells were proportionally decreased by L-765,314 in dose-dependently manner (Fig. 18) and 10 μM of L-765,314 downregulated PKC and tyrosinase activity of NHM without altering expression of proteins involved in melanogenesis (Fig. 19, 20). These results suggest the anti-melanogenic activity of L-765,314 is conserved in both human and

mouse melanocytes.

# Discussion

The hyperpigmentation treatment that has been regarded as a symbol of wealth and beauty in ancient times has been studied for a long time. Rice bran, red bean powder, and apricot seed powder have already been used for whitening from the Three Kingdoms period. Intensive field studies have developed and used vitamin C derivatives, placenta extracts, arbutin, Kojic acid and natural products such as yulmu and cucumber as a prophylaxis and treatment for hyperpigmentary disorders.

Previous studies have shown that increased PKC activity can elevate tyrosinase activity and upregulate melanin formation [13]. Park et. al showed that PKC-β directly modulates tyrosinase activity through phosphorylating serine residues of tyrosinase [25]. In addition, studies found drugs targeting protein expression of MITF and tyrosinase have been conducted [24]. There is also a report showing the correlation between melanogenesis and PKC activity [26]. PKA (cAMP signaling) which are mainly involved in melanogenesis have also been appeared to induce

melanogenesis by activating PKC [27].

In this study, melanin accumulation was also decreased in L-765,314 treated mouse melanocytes (Fig. 4). To confirm mechanism of L-765,314, we examined the expression level of protein and mRNA, however L-765,314 did not alter the expression of MITF, Tyrosianse, TRP1, TPR2 (Fig. 11, 12, 13). On the other hand, despite of comparable level of tyrosinase expression, we found that L-765,314 suppressed tyrosinase activity (Fig. 14). Western blot with a phospho-PKC substrate antibody, we confirmed that PKC activity was reduced by L-765,314 treatment and it is associated with reduction of tyrosinase activity (Fig. 15). In line with this, TPA treatment upregulated tyrosinase activity and melanin content, which was attenuated by pretreatment of L-765,314 (Fig. 15, 16). In addition, it was confirmed that melanin synthesis was also different (Fig. 17). Finally, we demonstrated that anti-melanogenic activity of L-765,314 was conserved in human melanocyte; decrease in PKC activity, and melanin production (Fig. 18, 19, 20).

Collectively, our study suggest that L-765,314 could contribute to the development of novel cosmetics and therapetics for the treatment of hyperpigmentation. However, since the scope of research using L-765,314 has been still limited, much research has to be pursued and molecular details of L-765,314 action on melanogenesis should be explored.

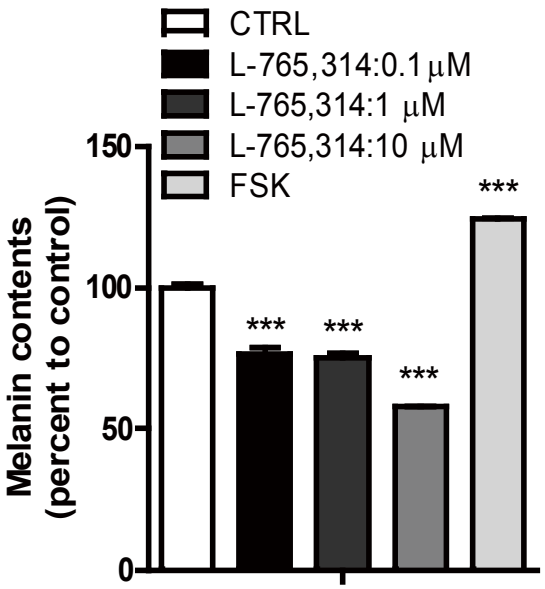


**Fig. 1 Changes of melanin production in B16-F10 cells with L-765,314 treatment.**

Melanin contents were measured by treating L-765,314 with 0.1 to 10 μM and FSK (10 μM) on B16-F10 cell. As a result, the amount of melanin was reduced by 24%, 25% and 42%, respectively. *** represents $p < 0.001$.

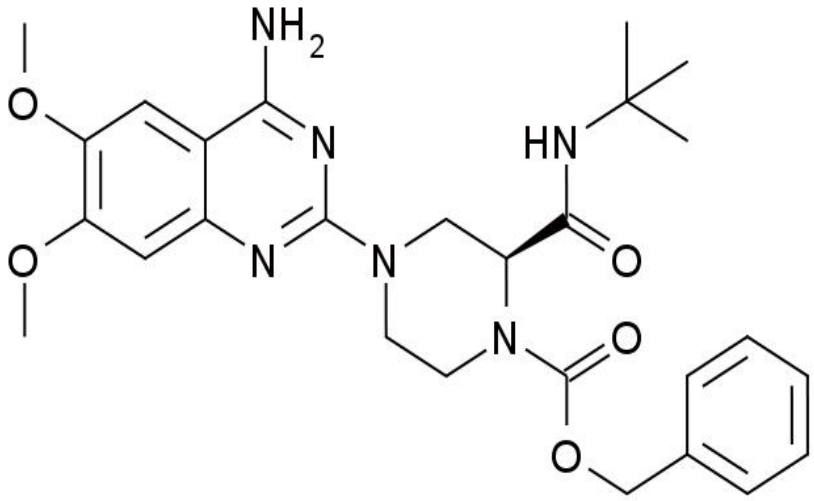


**Fig. 2 Structure of L-765,314 ((2S)-4-(4-amino-6,7-dimethoxy-2-quinazolinyl)-2-[[(1,1-dimethylethyl)amino]carbonyl]-1-piperazinecarboxylic acid).**

The constitutional formula of L-765,314.

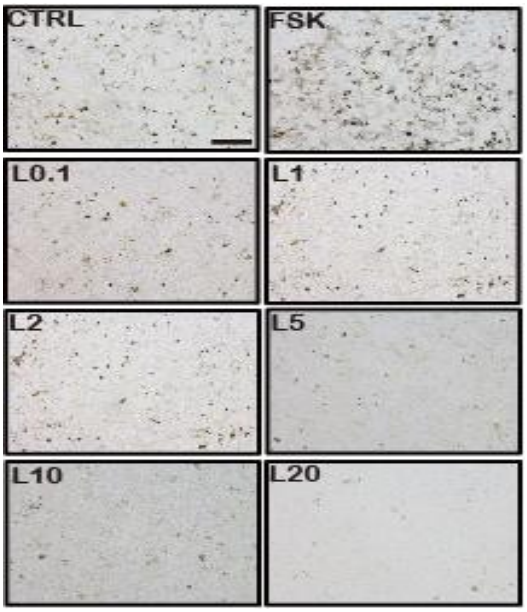


**Fig. 3 Microscopic images of Mel-Ab cells with L-765,314 treatment for 96 hours.**

Mel-Ab cells were treated with 0.1-20 uM of L-765,314 and melanin accumulation was tested by microscopy. FSK was used as a positive control to increase the production of melanin by activating cAMP signaling. Scale bar: 1000 μm

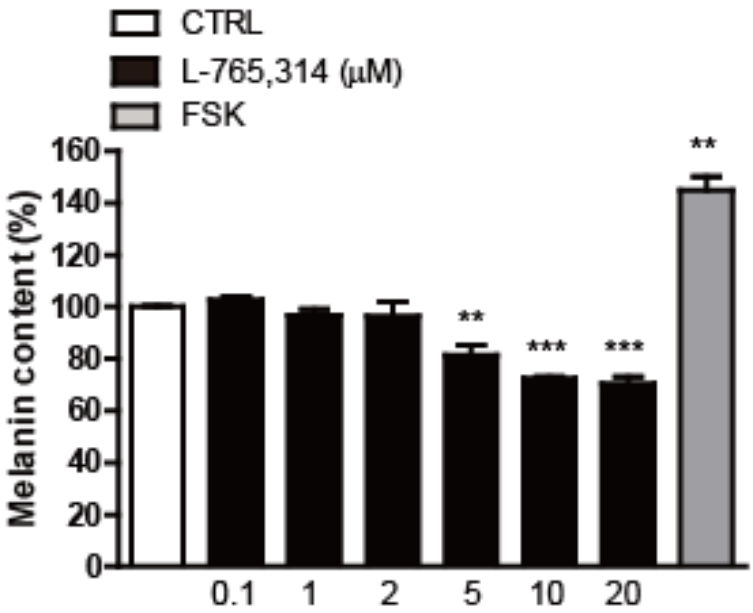


**Fig. 4 Reduction of melanin production in Mel-ab cells with L-765,314 treatment.**

Melanin production was measured after 0.1-20 uM of L-765,314 treatments in Mel-ab cells. Melanin expression were decreased by 28% at 10 μM and 30% at 20 μM of L-765,314. Whereas FSK treatment increased melanin production about 45% compared to vehicle treated Mel-Ab cells. ** and *** represents $p < 0.01$, and $p < 0.001$ respectively

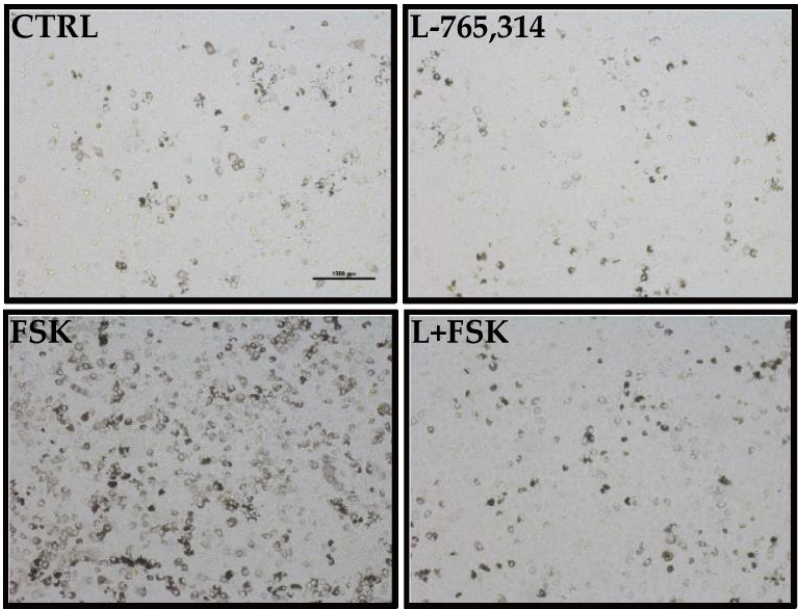


**Fig. 5 Microscopic observations of melanin production by treating L-765,314 with a positive control group.**

FSK was used as a positive control and L-765,314 (10 μM) were treated together to investigate the change of melanin production. The amount of melanin was significantly increased by FSK treatment. In contrast, melanin production decreased when L-765,314 pretreated. Scale bar: 1000 μm

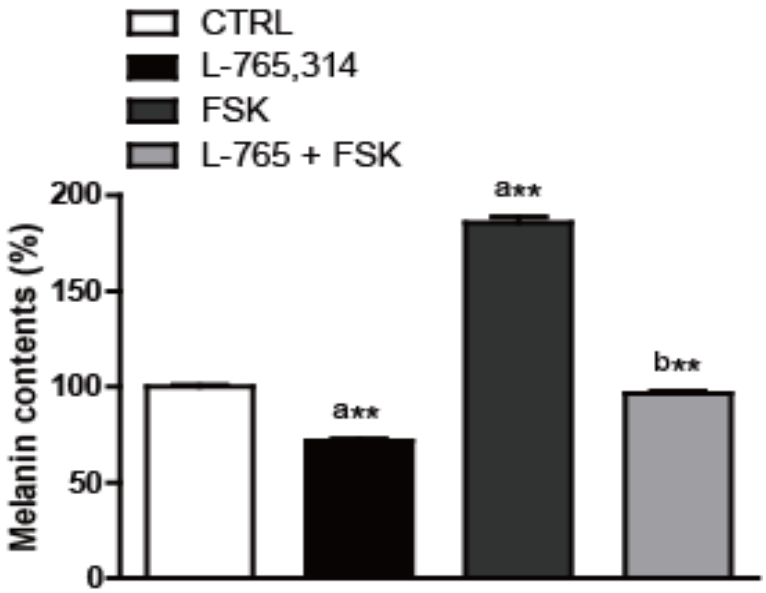


**Fig. 6 L-765,314 suppressed FSK-stimulated melanin production in Mel-ab cells.**

Examine the changes of melanogenesis with treatment of FSK or L-765,314 (10 μM) or together. As expected, L-765,314 inhibited the production of melanin by treating FSK. ** represents $p < 0.01$.

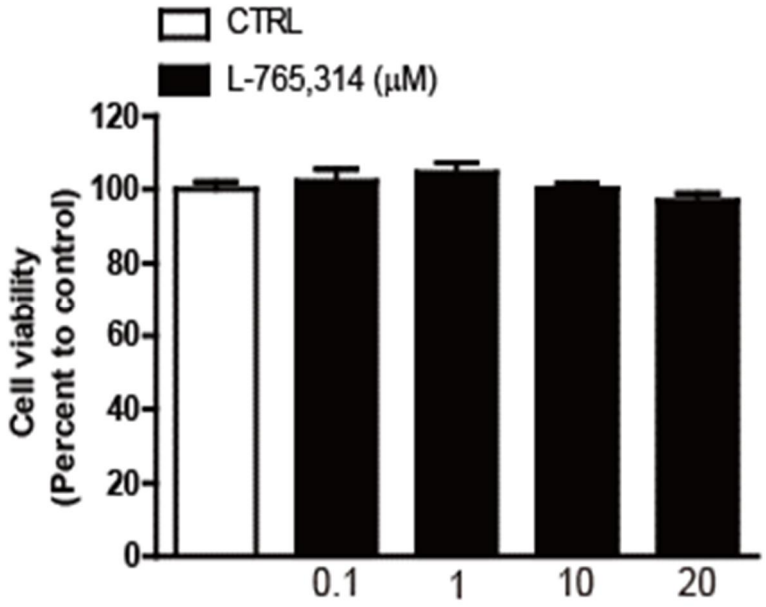


**Fig. 7 Investigation on cell viability according to the concentration of L-765,314 in Mel-ab cells.**

Examine the cytotoxicity of L-765,314 by MTT assay. Concentrations of L-765,314 up to 20 μM did not affect Mel-ab cell viability, suggesting that the anti-melanogenic effect of L-765,314 was not caused by nonspecific cytotoxicity.

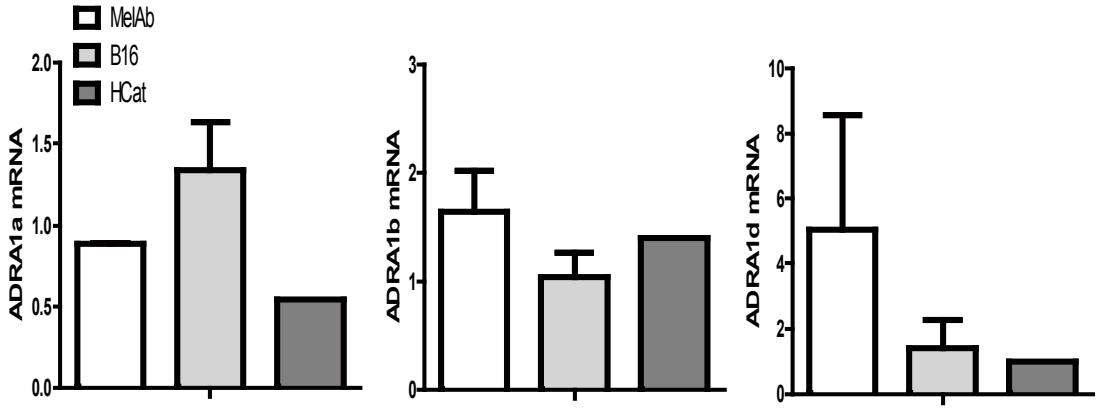


**Fig. 8 mRNA levels of ADRA1 subtype receptors in cells resident in epidermis of skin tissues.**

Expression of ADRA1a, ADRA1b, and ADRA1d in Mel-ab, B16-F10, and Human keratinocytes (HaCat) was analyzed by qRT-PCR. As a result, all of ADRA1 receptors are expressed in all three types of cells.

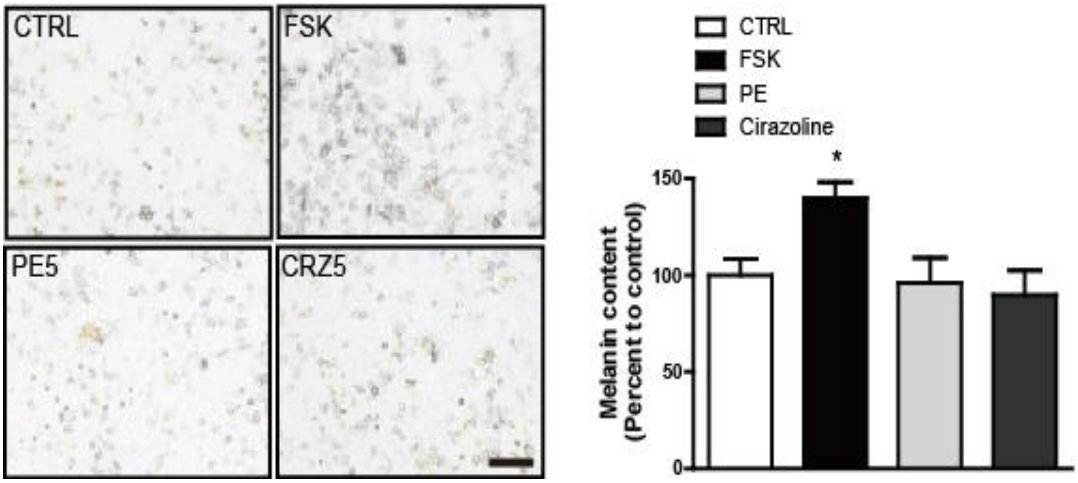


**Fig. 9 Investigation of melanogenesis changes after treatment with ADRA agonists at the usual concentrations in Mel-ab cells.**

Neither exposure to phenylephrine nor to cirazoline increased melanin content in Mel-ab cells. Phenylephinre is a selective ADRA agonist and cirazoline is a full ADRA1a agonist and partial agonist for ADRA1b. Each drugs were treated at 5 μM.

* represents $p < 0.05$. Scale bar: 500 μm

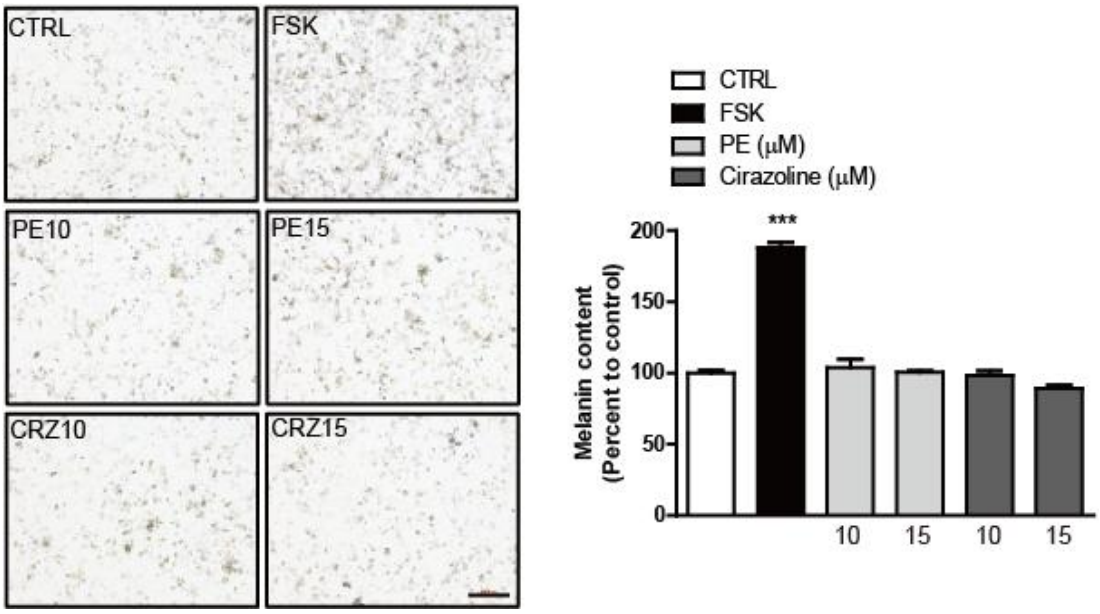


**Fig. 10 Investigation on changes in melanin production after treatment with high concentrations of ADRA agonists in Mel-ab cells.**

Phenylephrine and cirazoline were treated at a higher concentration (10 ~ 15 μM) than the previous experiment in Fig 9. As a result, the melanin expression was not increased as in the case of treatment with 5 μM. *** represents $p < 0.001$. Scale bar: 1000 μm.

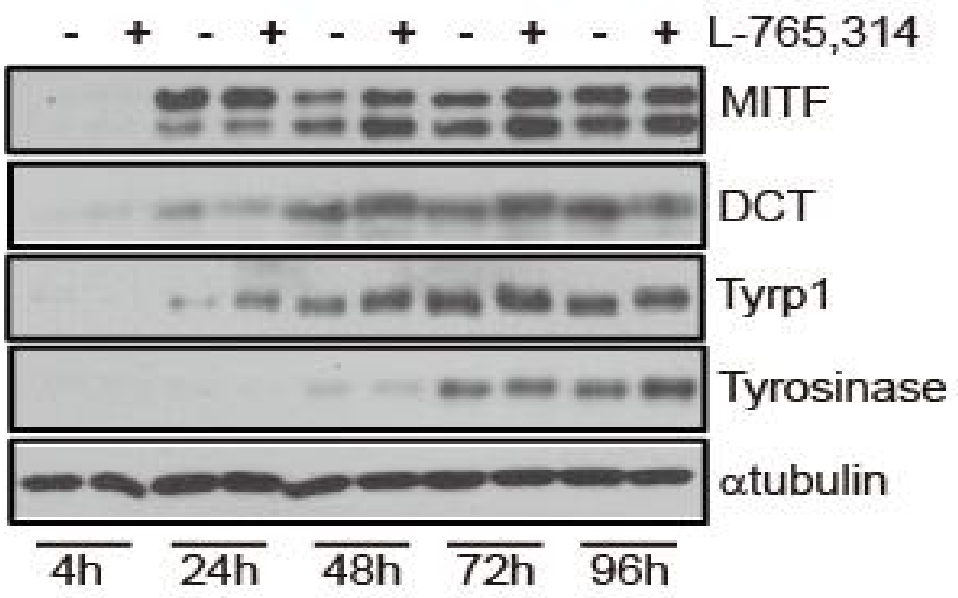


**Fig. 11 Investigation on protein expression with L-765,314 treatment in Mel-ab cells.**

The expression of MITF, Tyrosinase, TRP1 (=Tyrp1), and TRP2 (=DCT) proteins, which are involved in melanogenesis, was tested by Western blot. $\alpha$-tubulin was used as an internal loading control. As a result, L-765,314 (10 μM) didn't alter protein expressions.

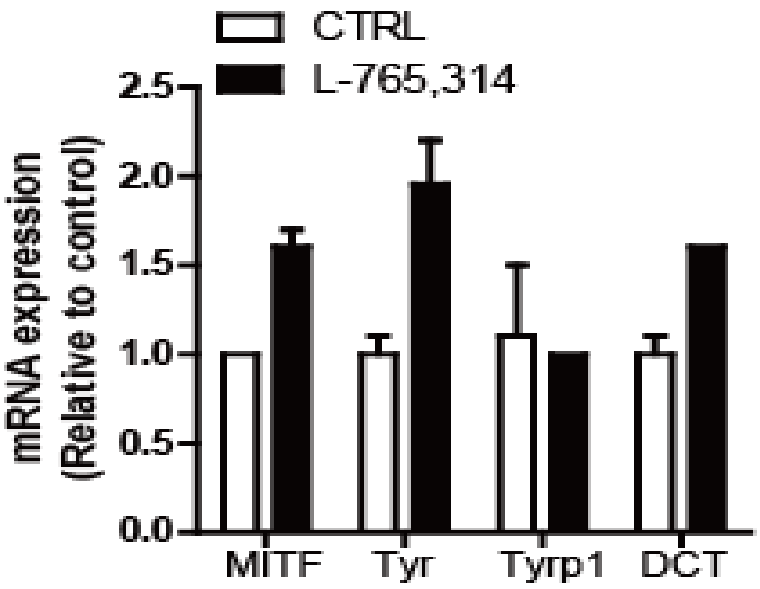


**Fig. 12 Investigation on RNA expression in Mel-ab cells with L-765,314 treatment for a certain time.**

The transcription levels of MITF, Tyrosinase, TRP1 (=Tyrp1), and TRP2 (=DCT) with 96 hours of L-765,314 (10 μM) treatment in Mel-Ab cells were compared to control cells by qRT-PCR. L32 was used as an internal control. As a result, as in the case of protein, L-765,314 does not alter melanogenic gene expression. Even some genes have increased.

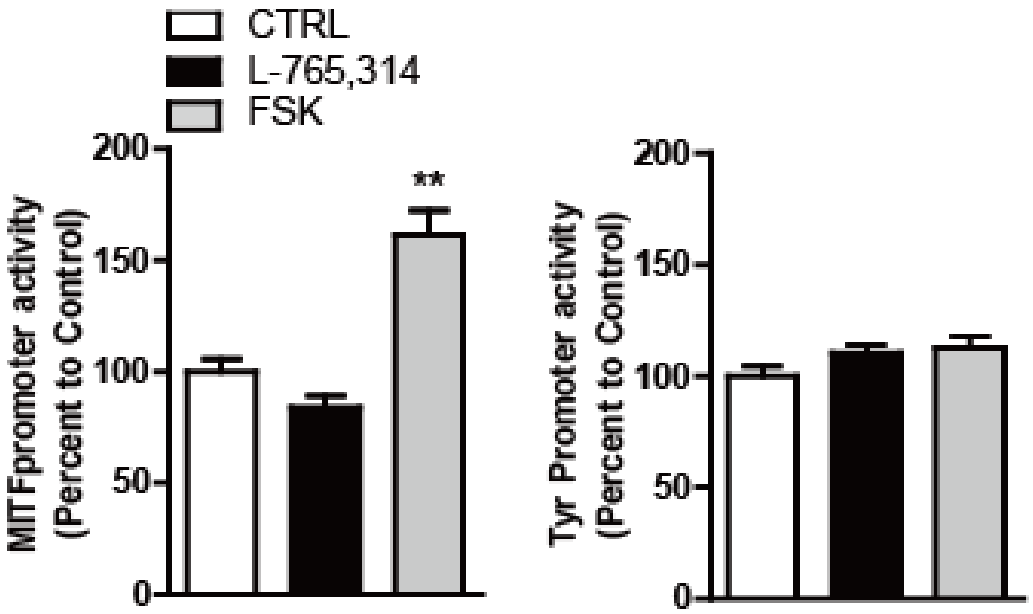


**Fig. 13 Investigation on promoter expression in Mel-ab cells with L-765,314 treatment.**

Because changes in protein and RNA levels of MITF, Tyrosinase, TRP1, and TRP2 were not clear, we tested luciferase assay for the effect of L-765,314 on MTTF and tyrosinase promoter activity. L-765,314 (10 μM) and FSK (10 μM) were treated for 6 hours. FSK was used as a positive control for enhancing MITF promoter activity. As a result, L-765,314 did not affect the expression of MITF and tyrosinase promoter. ** represents $p < 0.01$.

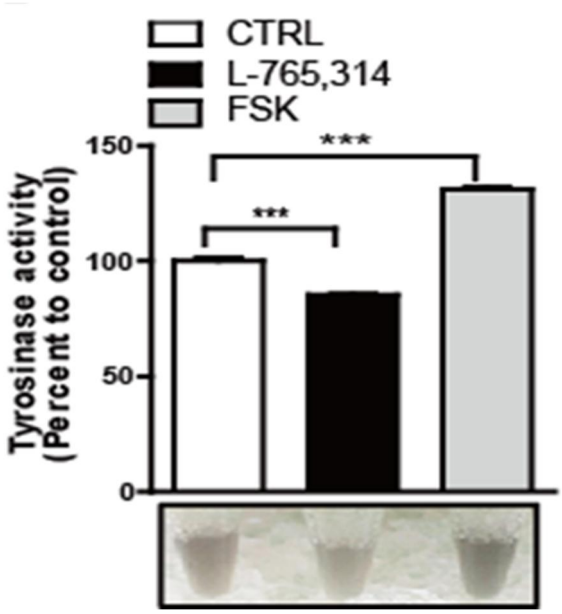


**Fig. 14 Investigation on tyrosinase activity after treatment with L-765,314 in Mel-ab cells.**

Because there was no difference in expression of the gene, we tested the tyrosinase activity in Mel-ab cells.tyrosinase is a rate-limiting enzyme on melanogenesis. Vehicle, L-765,314 (10 μM) and FSK (10 μM) were treated on Mel-ab cells to measure tyrosinase activity at 96 hours. As a result, the activity of tyrosinase decreased by 20% in Mel-ab cells with L-765,314 treatment for 96 hours. *** represents $p < 0.001$.

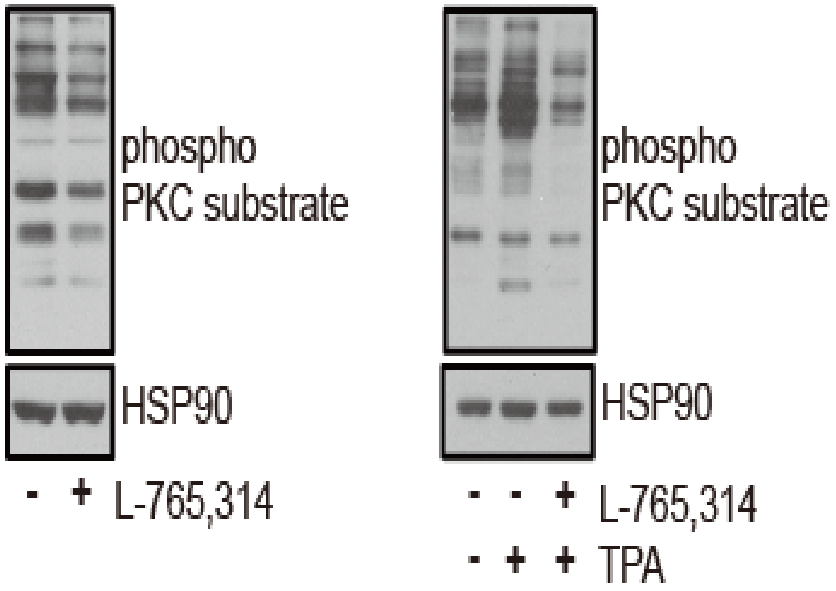


**Fig. 15 Investigation on PKC activity with L-765,314 and TPA-treated protein in Mel-ab cells.**

Because Protein kinase C (PKC) is known to affect the activity of tyrosinase, PKC activity was investigated in Mel-ab cells with L-765,314 (10 μM) treatment for 96 hours by western blot. Consequently, the expression of phospho PKC substrate decreased. Furthurmore, the PKC activity of Mel-ab cells was examined with vehicle, 12-O-Tetradecanoylphorbol 13-acetate (TPA, 100 nM), or L-765,314 and TPA. PKC activity was increased when TPA treatment only and decreased when TPA treatment with L-765,314 pretreated.

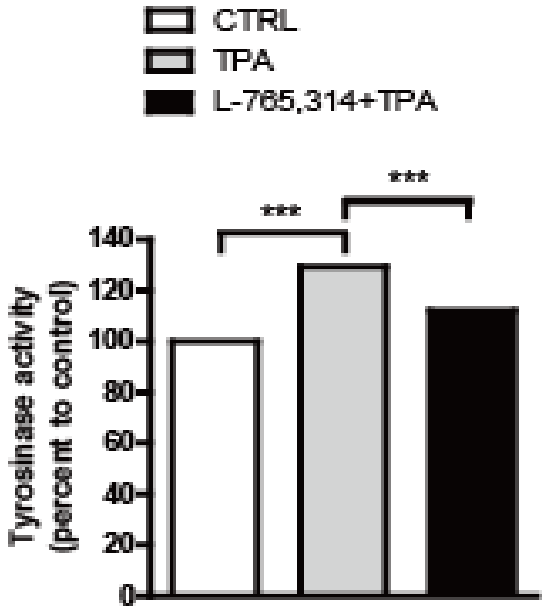


**Fig. 16 Investigation on tyrosinase activity after L-765,314 and TPA treatment in Mel-ab cells.**

The tyrosinase activity of Mel-ab cells was examined with vehicle, TPA (100 nM), or TPA with L-765,314 (10 μM) pretreatment. The tyorisnase activity increased when only TPA treated, but tyrosinase activity increasing rate decreased when TPA with L-765,314 pretreatment. *** represents $p < 0.001$.

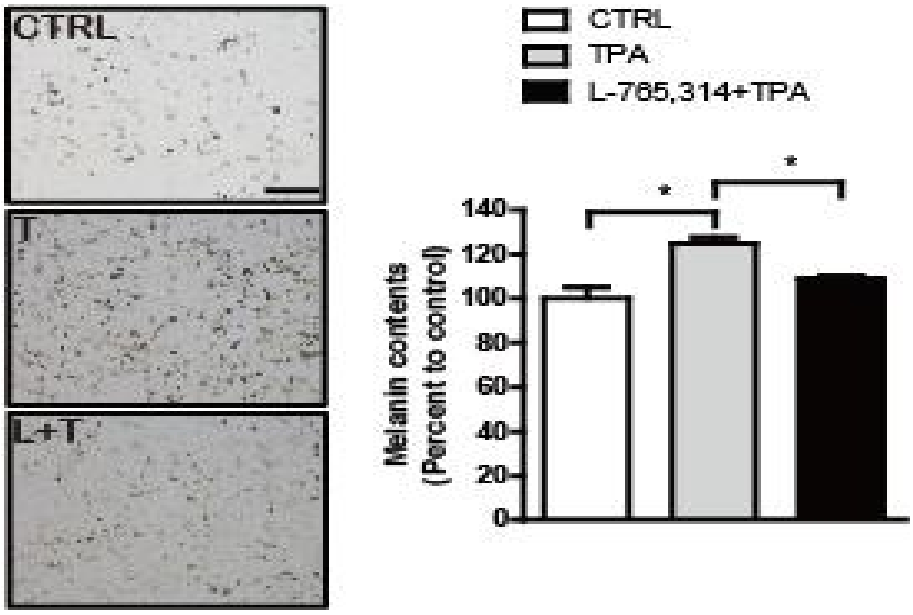


**Fig. 17 Investigation on changes in melanin production after L-765,314 and TPA treatment in Mel-ab cells.**

In order to determine the amount of melanin produced, we treated Vehicle, TPA (100 nM) and TPA with L-765,314 (10 μM) pretreatment in Mel-ab cells for 96 hours. The production of melanin decreased in the case of TPA with L-765,314 pretreatment than TPA alone. * represents $p < 0.05$.

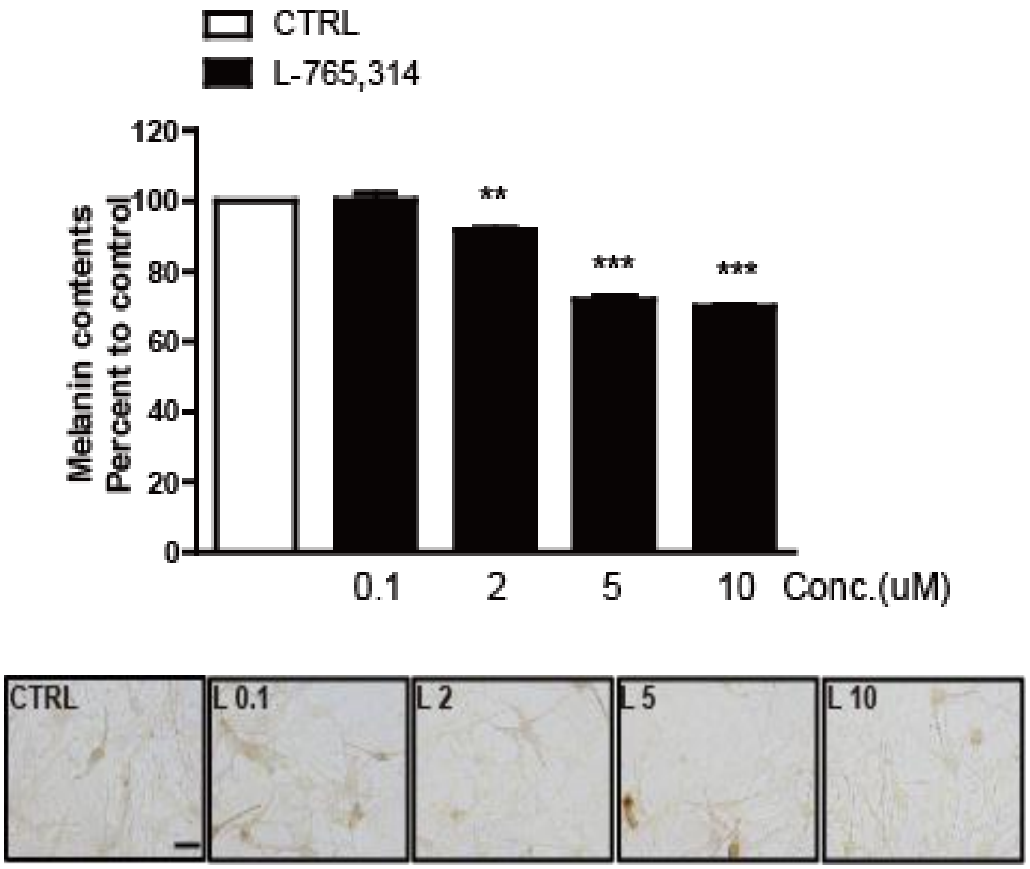


**Fig. 18 Changes of melanin production with L-765,314 treatment in normal human melanocytes (NHM).**

In order to measure the amount of melanin production, we treated L-765,314 from 0.1 μM to 10 μM for 96 hours in normal human melanocyte (NHM). In consequence, it was showed that as the treatment concentration of L-765,314 increased, the degree of melanin production decreased. ** and *** represents $p < 0.01$ and $p < 0.001$, respectively.

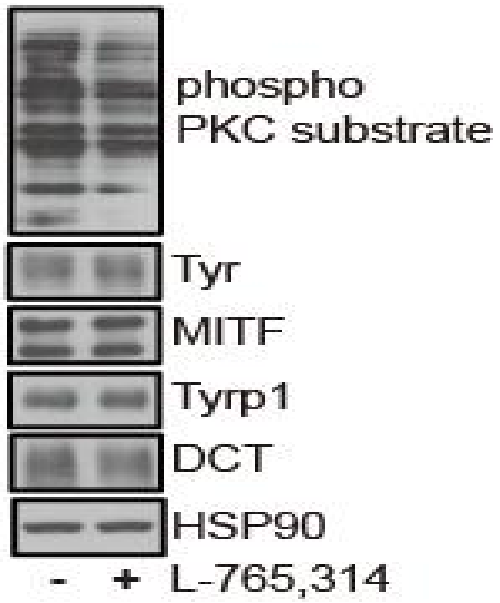


**Fig. 19 L-765,314 downregulated PKC activity on protein level in NHM cells.**

Western blot analysis of protein samples with L-765,314 (10 μM) treatment in NHM cells for 96 hours. L-765,314 didn't affect proteins related with melanogenesis but decreased PKC activity as same as in Mel-ab.

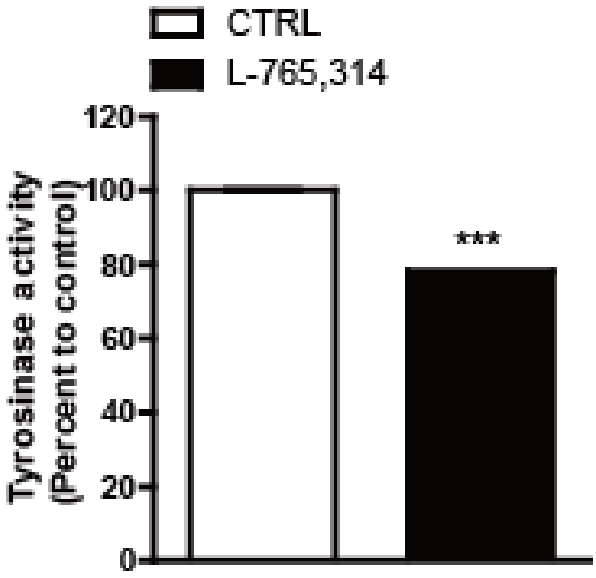


**Fig. 20 L-765,314 downregulated the tyrosinase activity in NHM cells.**

Finally, we measured tyrosinase activity in NHM cells with L-765,314 (10 μM) treatment for 96 hours. As a result, the tyrosinase activity was decreased about 20% by L-765,314. *** represents $p < 0.001$.

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# 국문요약

멜라닌의 적절한 형성은 강한 자외선으로 인한 일광 화상 및 DNA의 손상을 방지하는데 중요하다. 하지만 멜라닌 과형성으로 생기는 질환 또한 문제가 크다. 또한 미용적 측면에서 봤을 때 미백은 예전부터 관심도가 높은 분야 중 하나였다. 우리는 기존의 치료제가 보이는 피부자극, 염증, 각질, 홍진 및 가려움증 등의 부작용 때문에 새로운 약물을 찾기 위한 연구가 필요하다고 생각하였고 이에 B16-F10이라고 하는 mouse melanoma cells에서 멜라닌 생성을 억제하는 약물을 스크리닝 하여 L-765,314 (benzyl (2S)-4-(4-amino-6,7-dimethoxyquinazolin-2-yl)-2-(tert-butylcarbamoyl)-piperazine-1-carboxylate)를 발견하게 되었다. 하지만 L-765,314가 어떤 기전으로 멜라닌의 형성을 줄이는지에 대한 연구는 진행되어 있지 않았다. 따라서 본 연구는 L-765,314의 멜라닌 형성 과정에 작용하는 기전을 파악하기 위해 진행되었다. 우선 Mel-ab이라는 mouse melanocytes에 L-765,314를 농도 별로 처리하여 멜라닌 생성량을 확인한 결과 멜라닌의 발현이 감소함을 알 수 있었다. L-765,314의 독성을 확인하기 위하여 같은 방식으로 처리 하여 MTT

assay를 진행하였을 때 L-765,314는 세포의 사멸에는 관여하지 않는 것으로 나타났다. 웨스턴 블랏으로 확인한 결과 멜라닌 형성과 관련된 단백질의 발현도 변화가 없었다. MITF와 tyrosinase를 Luciferase assay로 확인해 본 결과 L-765,314는 MITF와 tyrosinase의 promoter 발현에 영향을 미치지 않았다. 하지만 tyrosinase activity를 확인해 봤을 때 L-765,314를 처리한 경우 tyrosinase activity가 감소하는 것이 확인되었다. 따라서 L-765,314는 단백질 형성과 관련된 단백질의 발현을 막는 것이 아니라 tyrosinase의 activity를 조절하여 멜라닌의 발현을 조절한다고 생각하게 되었다. 이를 뒷받침하기 위하여 L-765,314를 처리한 Mel-ab cells 단백질 샘플로 웨스턴 블랏을 진행하여 PKC activity를 확인해 보았다. Tyrosinase는 PKC가 활성을 조절하는 것으로 알려져 있기 때문이다. 그 결과 L-765,314를 처리할수록 PKC activity가 감소함을 확인할 수 있었다. 또한 human melanocytes에서 같은 방식으로 실험을 진행한 결과 역시 Mel-ab을 사용했을 때와 동일한 결과가 발견되었다. 이를 통해 L-765,314가 mouse와 human melanocytes에서 PKC의 activity를 조절하여 멜라닌 형성을 조절한다는 것을 확인하였다. 하지만 확실한 작용기전은 추가적인 연구를 진행해야 할 것으로 보인다.

중심단어: melanocytes / L-765,314 / melanogenesis / ADRA1b antagonist / PKC / tyrosinase activity