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1 **Alisol B, a triterpene from *Alismatis rhizoma* (dried rhizome of *Alisma*
2 *orientale*), inhibits melanin production in murine B16 melanoma cells**

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Abstract

To develop new whitening agents from natural products, we screened 80 compounds derived from crude drugs in Kampo medicine in a melanin synthesis inhibition assay using murine B16 melanoma cells. The screen revealed that treatment with alisol B, a triterpene from *Alismatis rhizoma*, significantly decreased both melanin content and cellular tyrosinase activity in B16 cells. However, alisol B did not directly inhibit mushroom tyrosinase activity *in vitro*. Therefore, we investigated the mechanism underlying the inhibitory effect of alisol B on melanogenesis. Alisol B suppressed mRNA induction of tyrosinase and its transcription factor, microphthalmia-associated transcription factor (MITF). Furthermore, alisol B reduced the phosphorylation of CREB and maintained the activation of ERK1/2. These results suggest that the reduction in melanin production by alisol B is due to the downregulation of MITF through the suppression of CREB and activation of ERK, and that alisol B may be useful as a new whitening agent.

Keyword: melanogenesis, alisol B, Kampo, tyrosinase, MITF

Abbreviations: IBMX, 3-isobutyl-1-methylxanthine; MITF, microphthalmia-associated transcription factor; CREB, cAMP-responsive element-binding protein; ERK, extracellular signal-regulated kinase; α-MSH, α-melanocyte-stimulating hormone; PKA, protein kinase A; MEK, mitogen-activated protein kinase kinase; RT-qPCR, reverse transcription quantitative real-time polymerase chain reaction.

Introduction

Melanin is synthesized in the melanosomes of melanocytes, and plays a crucial role in protecting the skin from the harmful effects of ultraviolet radiation.[1] Melanin synthesis or melanogenesis is mainly regulated by 3 melanogenic enzymes: tyrosinase (polyphenol oxidase; EC 1.14.18.10), tyrosinase-related protein-1 (TRP-1), and tyrosinase-related protein-2 (TRP-2). Tyrosinase is the rate-limiting enzyme in the melanogenic process, which catalyzes 2 different reactions: the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and oxidation of DOPA to dopaquinone.[2] In the absence of thiol substances, dopaquinone is first converted to dopachrome and then to 5,6-dihydoxyindole or indol-5,6-quinone 2-carboxylic acid (DHICA). TRP-2 catalyzes the conversion of dopachrome to DHICA, and TRP-1 catalyzes the oxidation of DHICA.[3] These melanogenic enzymes contain the consensus binding site for microphthalmia-associated transcription factor (MITF), a member of the basic helix-loop-helix-leucine zipper family of transcription factors, in their promoters, and are transcriptionally induced by MITF.[4]

α-Melanocyte-stimulating hormone (α-MSH), which is a tridecapeptide from the precursor proopiomelanocortin and belongs to the melanocortin family, binds and activates the G-protein-coupled melanocortin 1 receptor in melanocytes and in turn stimulates melanogenesis via the elevation of intracellular cAMP levels. cAMP-elevating agents such as forskolin (an adenylate cyclase activator) and 3-isobutyl-1-methylxanthine (IBMX) (a phosphodiesterase inhibitor) also stimulate melanin synthesis. An increase in intracellular cAMP levels leads to protein kinase A (PKA) activation, which subsequently phosphorylates the transcription factor cAMP-responsive element-binding protein (CREB) at Ser^{133}. The phosphorylated active form of CREB binds to the CRE motif of the MITF promoter and activates MITF transcription.[5] Thus, the cAMP/PKA/CREB pathway induces the expression of tyrosinase and its related proteins via MITF activation, thereby leading to the stimulation of melanogenesis.[6] On the other hand, the extracellular signal-regulated kinase (ERK) pathway is also involved in the regulation of melanin production.[7] Previous reports showed that the inhibition of ERK induced B16 melanoma cell differentiation and increased tyrosinase activity, and that the inhibition of mitogen-activated protein kinase kinase (MEK) activity by anthrax lethal toxin induced melanin production in human melanoma cells.[7, 8] In addition, it has been suggested that ERK activation leads to the phosphorylation and

1 degradation of MITF, resulting in a reduced tyrosinase level and decreased melanogenesis.[9)]

2 Because excessive production of melanin and its accumulation in the epidermis cause abnormal
3 hyperpigmentation of the skin, such as melisma, freckles, and senile lentigines, the development of
4 melanogenesis inhibitors has been focused. Kojic acid and arbutin have been often used as cosmetic
5 agents for skin whitening; however, they are associated with some problems. For example, kojic acid
6 causes allergic reactions such as contact dermatitis and sensitization if used for long stretches of time.[10, 11)]
7 Therefore, the discovery of safer and more effective whitening agents is required, and some potent
8 tyrosinase inhibitors, including luteolin, oxyresveratrol, and quercetine, have been identified from natural
9 plants.[12-14)] Glabridin, an isoflavane isolated from the roots of licorice (*Glycyrrhiza glabra*), also inhibits
10 tyrosinase activation, and is currently used as a skin-whitening ingredient. Licorice is one of the most
11 frequently-used crude drugs in Kampo medicines (traditional Japanese herbal medicines). Although many
12 crude drug components have been identified, it is not yet fully understood whether any of these
13 components can inhibit melanin biosynthesis. Therefore, we investigated the potential anti-melanogenic
14 effects of 80 compounds derived from crude drugs in Kampo medicines. We identified alisol B, a
15 triterpene extracted from *Alismatis rhizoma*, as a novel melanogenesis inhibitor in murine B16 melanoma
16 cells.

17

18 **Materials and methods**

19 *Cell culture.* Murine B16 melanoma cells were cultured in DMEM supplemented with 10% fetal
20 bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in 5% CO_2.

21

22 *Cell viability assay.* B16 cells were seeded at a density of 5 x 10^3 cells per well in 96-well culture
23 plates. After 24 hours, the cells were treated with 10 μM natural products or dimethyl sulfoxide (DMSO)
24 in the presence of 20 μM forskolin and 100 μM IBMX for 72 hours. All natural products were dissolved
25 in 100% DMSO at a concentration of 10 mM, and the final concentration of DMSO in the culture
26 medium was 0.1%. The relative viable cell number was determined by the 3-(4, 5-dimethylthiazol-2yl)-2,
27 5-diphenyltetrazolium bromide (MTT) assay. After a 2-hour incubation with MTT, the resulting formazan
28 crystals were dissolved in DMSO. The absorbance at 570 nm was determined using the Infinite M200

plate reader (TECAN Japan).

Measurement of melanin contents. B16 cells were seeded at a density of 2 x 10^4 cells into 24-well culture plates. After 24 hours, cells were pretreated with 10 μM natural products or DMSO for 30 min and stimulated with 20 μM forskolin and 100 μM IBMX. After 72 hours, cells were washed with ice-cold PBS and lysed with 1 N NaOH at 100 °C for 1 hour. After centrifugation at 16,000 x *g* for 20 min, the absorbance of the supernatant was measured at 405 nm.

Assay of cellular and in vitro tyrosinase. Tyrosinase activity was assessed by determining the catalysis of L-DOPA to dopachrome. To measure cellular tyrosinase activity, B16 cells were seeded at a density of 2 x 10^4 cells in 24-well culture plates. After 24 hours, cells were pretreated with 1 μM alisol B (Wako Pure Chemical Industries, Japan) or DMSO for 30 min, followed by stimulation with 20 μM forskolin/100 μM IBMX or 0.1 μM α-MSH (GenScript, purity > 95%) for 72 hours. The cells were lysed with 100 mM sodium phosphate buffer (pH 6.8) containing 1% Triton X-100, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. After centrifugation at 10,000 x g for 10 min, aliquot (100 μl) of the supernatant was mixed with 100 μl of a substrate solution containing100 mM sodium phosphate buffer (pH 6.8) and 0.5 mM L-DOPA, and incubated for 30 min at 37 °C. To monitor the production of dopachrome, the absorbance at 475 nm was measured using the microplate reader.

Reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) and immunoblotting. For RT-qPCR analysis, total RNA extracted from B16 cells was reverse transcribed using ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO, Japan). Quantitative real-time PCR was conducted using ABI Prism 7000 Sequence Detection System (Applied Biosystems) with Power SYBR Green PCR Master Mix (Applied Biosystems) as previously described.[15)] The following specific primers were used: tyrosinase, forward 5'-GTCGTCACCCTGAAAATCCTAACT-3' and reverse 5'-CATCGCATAAAACCTGATGGC-3'; MITF, forward 5'-GTATGAACACGCACTCTCGA-3' and reverse 5'-GTAACGTATTTGCCATTTGC-3'; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward 5'- GTGTCCGTCGTGGATCTGA -3' and reverse 5'-

CCTGCTTCACCACCTTCTTG -3'. GAPDH was used as an internal control.

For immunoblotting, cells were lysed with lysis buffer [20 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 10 μg/ml aprotinin, and 10 μg/ml leupeptin]. After centrifugation at 10,000 × *g* for 10 min, the supernatants were performed for immunoblotting with antibodies against CREB, phospho-CREB, ERK1/2, phospho-ERK1/2, MEK1/2, phospho-MEK1/2 (Cell Signaling Technology), MITF, or β-actin (SantaCruz Biotechnology).

Statistical analysis. All experiments were performed multiple times to confirm their reproducibility. One representative set of data was shown in the figures. Immunoblot band intensities were quantified using Image J software (NIH). Data were expressed as the mean ± standard error, and statistical analysis was performed by Student's t-test or one-way analysis of variance (ANOVA) with Tukey's multiple comparison test using GraphPad Prism (GraphPad Software).

Results

Identification of melanogenesis inhibitors from a compound library derived from crude drugs in Kampo medicine

We first screened 80 chemically defined natural products in traditional Japanese herbal medicines (Kampo medicines) for their ability to inhibit melanin pigmentation in murine B16 melanoma cells. To induce melanin pigmentation, B16 cells were stimulated with a combination of 20 μM forskolin and 100 μM IBMX for 72 hours, resulting in a significant increase (> 2-fold) in melanin production (Fig. 1A). After pretreatment with the compounds at a concentration of 10 μM, cellular melanin contents in forskolin/IBMX-stimulated cells were examined. Among the 80 compounds that we tested, 8 compounds (alisol B, berberine chloride, bergenin, coptisine chloride, corydaline, glabridin, loganin, and nodakenin) apparently decreased melanin pigmentation stimulated with forskolin/IBMX (data not shown). Therefore, we measured melanin contents in B16 cells treated with forskolin/IBMX in the presence and absence of these 8 compounds. As shown in Fig. 1A, treatment with alisol B, berberine chloride, coptisine chloride, corydaline, or glabridin at 10 μM significantly inhibited the increase in melanin production stimulated with forskolin/IBMX. Next, we examined the cytotoxicity of these compounds in B16 cells by the MTT

1 assay. B16 cells were treated with each compound at 10 μM in the presence of forskolin/IBMX for 72
2 hours. As shown in Fig. 1B, berberine chloride, coptisine chloride, and nodakenin had cytotoxic effects
3 on cells, but alisol B and glabridin did not affect cell viability. Because glabridin, a major active
4 flavonoid in *G. glabra*, has been reported to possess anti-melanogenic activity via the inhibition of
5 tyrosinase,[16] alisol B was chosen as a candidate anti-melanogenic agent (Fig. 1C). The percent inhibition
6 of melanin production by alisol B at 0.1 μM, 1 μM, and 10 μM was 15%, 69%, and 94%, respectively,
7 and its 50% inhibitory concentration (IC_{50}) was 440 nM (data not shown). This result indicates that alisol
8 B clearly exhibits enough inhibitory activity to reduce melanin production in B16 cells at a concentration
9 of 1 μM. Therefore, for further experiments, alisol B was used at a concentration of 1 μM.
10
11 *Effect of alisol B on melanin production and tyrosinase activity in B16 cells*
12 Alisol B is one of the major triterpene constituents and is known a bioactive component of *A. rhizoma*
13 (dried rhizome of *Alisma orientale*).[17] Although *A. rhizoma* is well-known in Chinese traditional
14 medicine and has been used to treat diuresis and hyperlipidemia,[18] no previous study has examined its
15 anti-melanogenic activity. Therefore, we examined whether *A. rhizoma* reduces melanin synthesis
16 stimulated with forskolin/IBMX. In addition, we tested the effect of *A. rhizoma* and alisol B on
17 α-MSH-induced melanin pigmentation. B16 cells pretreated with alisol B or *A. rhizoma* were stimulated
18 with forskolin/IBMX or α-MSH for 72 hours, following which their melanin content was measured. As
19 shown in Fig. 2A and B, stimulation with forskolin/IBMX and α-MSH resulted in an approximately
20 2-fold increase in melanin content compared with the control. Alisol B efficiently decreased melanin
21 production induced by both forskolin/IBMX and α-MSH compared with glabridin, a potent tyrosinase
22 inhibitor. On the other hand, *A. rhizoma* had no or little influence on the production of melanin in any
23 stimulated cells, suggesting the existence of components that counteract the effect of alisol B.
24 Because tyrosinase is the rate-limiting enzyme for melanin synthesis, we next examined the inhibitory
25 effect of alisol B on cellular tyrosinase activity in forskolin/IBMX- or α-MSH-stimulated B16 cells. As
26 shown in Fig. 2C and D, B16 cells showed increased tyrosinase activity upon exposure to
27 forskolin/IBMX and α-MSH. Alisol B significantly suppressed both forskolin/IBMX- and
28 α-MSH-induced cellular tyrosinase activity. Furthermore, we measured the inhibitory effect of alisol B on

in vitro mushroom tyrosinase inhibition assay using L-DOPA as a substrate. Alisol B (final concentration at 1 μM and 10 μM) had no inhibitory effect on mushroom tyrosinase activity, although reduced glutathione, a compound that inhibits tyrosinase activity, significantly inhibited (data not shown). These results suggest that the inhibition of melanin synthesis by alisol B is accompanied by a parallel decrease in tyrosinase activity, perhaps by the suppression of tyrosinase expression.

Effect of alisol B on expression of tyrosinase and MITF

We elucidated whether alisol B influences the mRNA expression of tyrosinase. Real-time quantitative PCR analysis revealed that forskolin/IBMX treatment dramatically increased the mRNA level of tyrosinase, and that its induction was significantly inhibited by alisol B (Fig. 3A). Furthermore, the mRNA expression of MITF, a key transcription factor that regulates the expression of the tyrosinase gene, was also measured. As shown in Fig. 3B, alisol B slightly but significantly reduced the induction of MITF mRNA by forskolin/IBMX. In addition, B16 cells pretreated with alisol B were stimulated with forskolin/IBMX for 30 min or 60 min, and the level of MITF protein in these cells was determined by immunoblot analysis. Fig. 3C shows that MITF protein level was decreased by pretreatment with alisol B. These results indicate that alisol B represses tyrosinase expression partially via the reduction of MITF mRNA and protein levels, leading to the down-regulation of melanin synthesis.

Effects of alisol B on the signaling pathway involved in melanogenesis

MITF expression is induced by CREB activated through phosphorylation at Ser^{133}.[19] Thus, we performed immunoblot analysis to determine whether alisol B affects CREB phosphorylation. As shown in Fig. 4A, the phosphorylation of CREB at Ser^{133} was hardly detectable in unstimulated B16 cells, but was increased at 30 min and further increased at 60 min after stimulation with forskolin/IBMX. Compared with untreated cells, alisol B remarkably decreased CREB phosphorylation by 61% after stimulation for 60 min, although it had no effect on CREB phosphorylation after 30 min. This indicates that alisol B downregulates MITF expression partially through the inhibition of CREB phosphorylation. In addition to the cAMP/PKA/CREB pathway, the mitogen-activated protein kinase (MAPK) signaling pathway is also involved in melanogenesis.[7, 8, 20] The activation of ERK MAPK leads to MITF

ubiquitination and degradation, and thus inhibits melanin synthesis.[9, 21] Therefore, we investigated whether alisol B influences ERK activation. After forskolin/IBMX stimulation, ERK1/2 was transiently phosphorylated with a peak at 30 min, and then diminished (Fig. 4B). Pretreatment with alisol B induced prolonged phosphorylation of ERK1/2, which remained strongly phosphorylated after 60 min. Similarly, the phosphorylation of MEK1/2, an upstream kinase of ERK, was also sustained by alisol B treatment (Fig. 4C). Finally, we examined whether the inhibition of the MEK/ERK pathway affects MITF reduction by Alisol B. B16 cells were treated with Alisol B in the presence or absence of a MEK-specific inhibitor U0126, followed by stimulation with forskolin/IBMX for 30 min. As shown in Fig. 4D, MITF protein level was significantly reduced by alisol B and the reduction was slightly recovered by U0126. These results indicate that alisol B plays a dual role, namely the inhibition of the cAMP/PKA/CREB pathway and activation of the MEK/ERK pathway, thereby inducing the downregulation of MITF.

Discussion

The development of natural products from various plant sources that inhibit melanin formation and tyrosinase activity has been actively pursued, because the existing skin-whitening products have been reported to exhibit some side effects. To develop safer and more efficient pharmaceutical and cosmetic products, we focused on chemically defined natural products in Kampo medicines, which are traditional Japanese herbal medicines. In the present study, we identified alisol B [(23S,24R)-24,25-epoxy-11b,23-dihydroxy-8a,9b,14b-dammar-13(17)-en-3-one] as an anti-melanogenic compound, whose activity is higher than that of a known tyrosinase inhibitor, glabridin. Alisol B is one of the major active constituents of *A. rhizoma*, which also contains alisol A, C, and their related compounds as active triterpenoids.[17] Although *A. rhizoma* has been used because of its diuretic, hypolipidemic, and anti-inflammatory properties,[18] its anti-melanogenic activity has not been studied. We found that *A. rhizoma* has little anti-melanogenic activity. Furthermore, alisol A [(23S,24R)-11b,23,24,25-tetrahydroxydammar-13(17)-en-3-one], which was also tested in this study, had a much lower inhibitory effect than alisol B (27%-55% alisol A vs. 64%-75% alisol B inhibitory activity at 10 μM) (data not shown). A previous study revealed that alisol B induces autophagy via the inhibition of the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase pump, whereas alisol A exhibits a much lower

inhibitory effect.[22] These results suggest that a slight structural difference between alisol A and B may exert a distinct influence on the physiological function.

Although alisol B significantly inhibited cellular tyrosinase activity stimulated with forskolin/IBMX and α-MSH, it (even at 10 μM) did not affect tyrosinase activity in an *in vitro* system using mushroom tyrosinase. Because tyrosinase is the rate-limiting enzyme in the melanogenic process, most of the strategies for the development of skin-whitening agents are based on the inhibition of tyrosinase activity. In fact, tyrosinase inhibitors, such as kojic acid and arbutin, are used in cosmetic products and as depigmenting agents for hyperpigmentation. On the other hand, the suppression of tyrosinase gene expression is also a target for the development of skin-whitening products. A recent report has demonstrated that hispolon, a compound of *Phellinus linteus*, inhibits melanogenesis through the down-regulation of tyrosinase and MITF expression.[23] The inhibitory effect of alisol B on melanogenesis also seems to be attributed to the suppression of tyrosinase gene expression rather than to the direct inhibition of tyrosinase activity.

The promoters of tyrosinase, TRP-1, and TRP-2 possess the consensus binding site for MITF, which is a master regulator of melanocyte development, and their expression is activated by MITF. Although alisol B significantly reduced the induction of tyrosinase and MITF mRNA by forskolin/IBMX, it did not affect the expression of TRP-1 and TRP-2 mRNA (data not shown). Chloroform extracts of fermented *Viola mandshurica* inhibited the mRNA expression of tyrosinase and MITF, but not of TRP-1 and TRP-2, in B16 cells.[24] On the other hand, acetoside, a phenylpropanoid glycoside isolated from the leaves of *Rehmannia glutinosa*, downregulated the expression of tyrosinase and TRP-1, but not of TRP-2.[25] This suggests that molecules other than MITF, for example, other transcription factors including repressors, may be also involved in the expression of tyrosinase and tyrosinase-related proteins, and that their enzymes may exhibit different expression patterns depending on the compounds.

MITF protein levels are regulated by protein kinase signaling pathways such as PKA and ERK MAPK. The cAMP/PKA pathway stimulates MITF promoter activity through the phosphorylation and activation of CREB, leading to an increase in MITF protein content. On the other hand, sustained ERK activation induces MITF phosphorylation and its subsequent degradation. Alisol B decreased the phosphorylation of CREB and continued to activate the MEK/ERK pathway, resulting in the reduction of MITF and

1 subsequent reduction of tyrosinase. In addition, alisol B had no effect on the activation of raf-1, which is
2 an upstream positive modulator of the MEK/ERK pathway (data not shown), suggesting that raf-1 is not a
3 target for alisol B. These results show that alisol B probably diminishes melanogenesis via two signaling
4 pathways: the PKA-CREB-MITF pathway and the MEK-ERK-MITF pathway.
5 In conclusion, we revealed that alisol B decreases MITF level through the regulation of the CREB and
6 ERK pathways in B16 melanoma cells, leading to the inhibition of tyrosinase expression, and
7 subsequently melanin production. These findings strongly suggest that alisol B can become a useful
8 therapeutic agent for the treatment of hyperpigmentation, and can be used as an ingredient in whitening
9 and lightening cosmetics. However, further research is required to evaluate the efficacy and safety of
10 alisol B.
11
12 **Disclosure statement**
13 No potential conflict of interest was reported by the authors.
14
15 **Author contribution**
16 IY, NY, and KY conceived and designed the experiments. IY and CI performed the experiments. IY,
17 CI, and KY analyzed data. AT, NY, and KY contributed reagents/materials/analysis tools. IY, AT, and
18 KY wrote the paper.
19
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23

Figure legends

Fig. 1. Identification of alisol B as an anti-melanogenic inhibitor**.**

Notes: (A, B) After pretreatment with 8 compounds (10 μM) (*Ctrl*; 0.1% DMSO, *1*; alisol B, *2*; berberine chloride, *3*; bergenin, *4*; coptisine chloride, *5*; corydaline, *6*; glabridin, *7*; loganin, *8*; nodakenin), B16 melanoma cells were stimulated with a combination of forskolin (20 μM) and IBMX (100 μM) for 72 hours. (A) The intracellular melanin content in B16 cells was measured as described under "Materials and methods". (B) The cell viability was quantified by the MTT assay. Results are expressed relative to forskolin/IBMX-treated cells (*closed bar*) (=100%). Values are the means ± S.E. for at least four cultures, and statistical analysis was performed by one-way ANOVA with Tukey's multiple comparison test. *$p<0.05$, **$p<0.01$, and ***$p<0.001$ compared with forskolin/IBMX stimulation. (C) Chemical structure of alisol B.

Fig. 2. Effect of alisol B and *A. rhizoma* on melanogenesis in B16 cells.

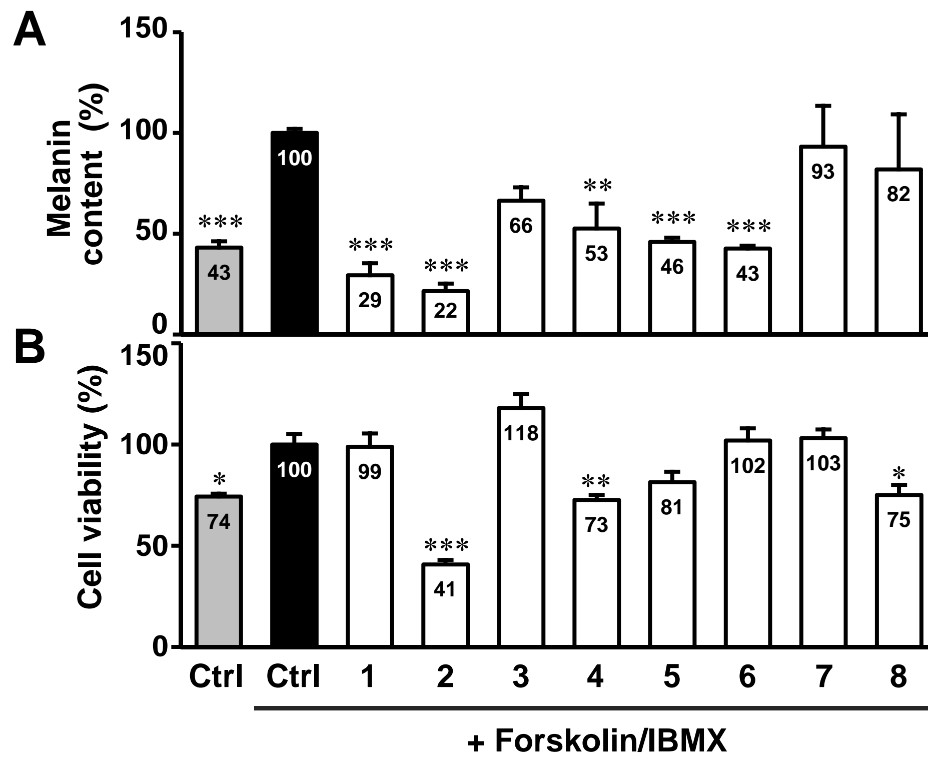
Notes: After pretreatment with DMSO (*Ctrl*), glabridin (1 μM), alisol B (1 μM), or *A. rhizoma* (10 μg/ml), B16 cells were stimulated with forskolin (20 μM) and IBMX (100 μM) (A, C) or with α-MSH (0.1 μM) (B, D) for 72 hours, following which melanin content (A, B) and tyrosinase activity (C, D) were measured as described under "Materials and methods". The results are expressed as means ± S.E. of three separate experiments, and statistical analysis was performed by one-way ANOVA with Tukey's multiple comparison test. **$p<0.01$ and ***$p<0.001$ compared with forskolin/IBMX or α-MSH stimulation.

Fig. 3. Inhibition of forskolin/IBMX-induced expression of tyrosinase and MITF by alisol B.

Notes: (A, B) B16 cells were preincubated with alisol B (1 μM), and treated with forskolin/IBMX for 72 hours. The mRNA expression level of tyrosinase (A) and MITF (B) was measured by real-time PCR analysis and was normalized to that of GAPDH. (C) B16 cells pretreated with alisol B were stimulated by forskolin/IBMX for 30 min and 60 min. The cell lysates were analyzed by immunoblotting using anti-MITF and anti-β-actin antibodies. The results are expressed as means ± S.E. of three separate experiments, and statistical analysis was performed by one-way ANOVA with Tukey's multiple comparison test. ***$p<0.001$ compared with forskolin/IBMX stimulation.

Fig. 4. Alisol B suppressed forskolin/IBMX-induced melanin production through the regulation of CREB and MEK/ERK.

Notes: B16 cells pretreated with alisol B were stimulated by forskolin/IBMX for 30 min and 60 min. The cell lysates were analyzed by immunoblotting using antibodies against CREB and phospho-CREB (A), ERK1/2 and phospho-ERK1/2 (B), and MEK1/2 and phospho-MEK1/2 (C). The levels of phosphorylated forms were normalized to the levels of total proteins. (D) B16 cells were pretreated with 1 μM alisol B in the presence or absence of 10 μM U0126 for 30 min, followed by stimulation with forskolin/IBMX for 30 min. The cell lysates were analyzed by immunoblotting using anti-MITF and anti-β-actin antibodies. The results are expressed as means ± S.E. of three separate experiments. *$p<0.05$ and **$p<0.01$ compared with forskolin/IBMX stimulation.



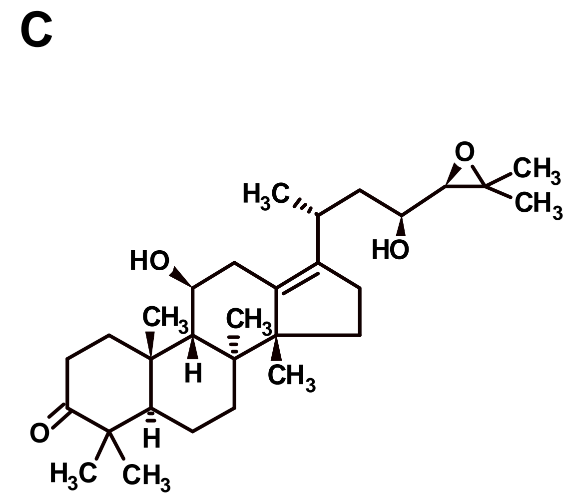
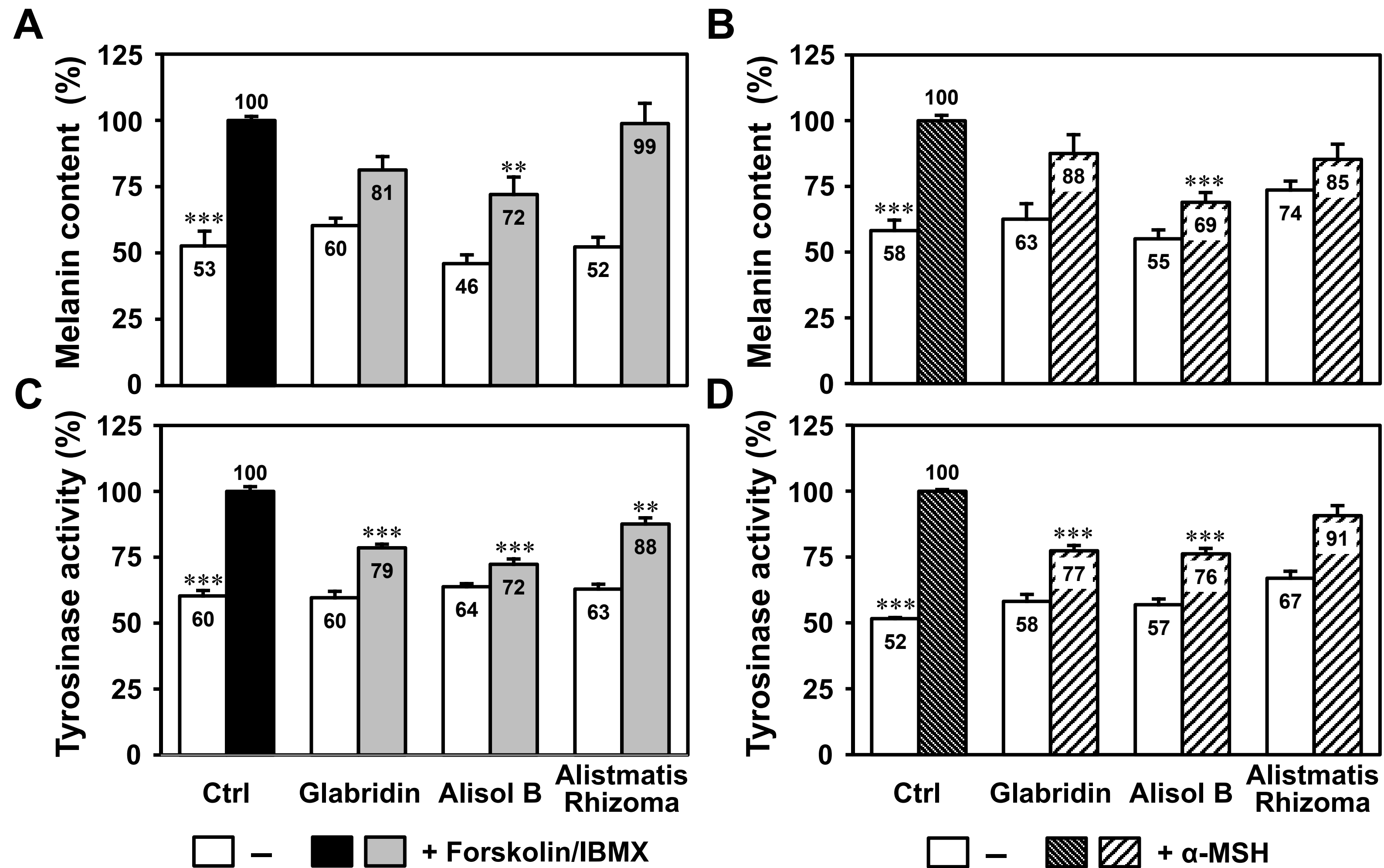


Fig.1. Yoshida et al. (2016)



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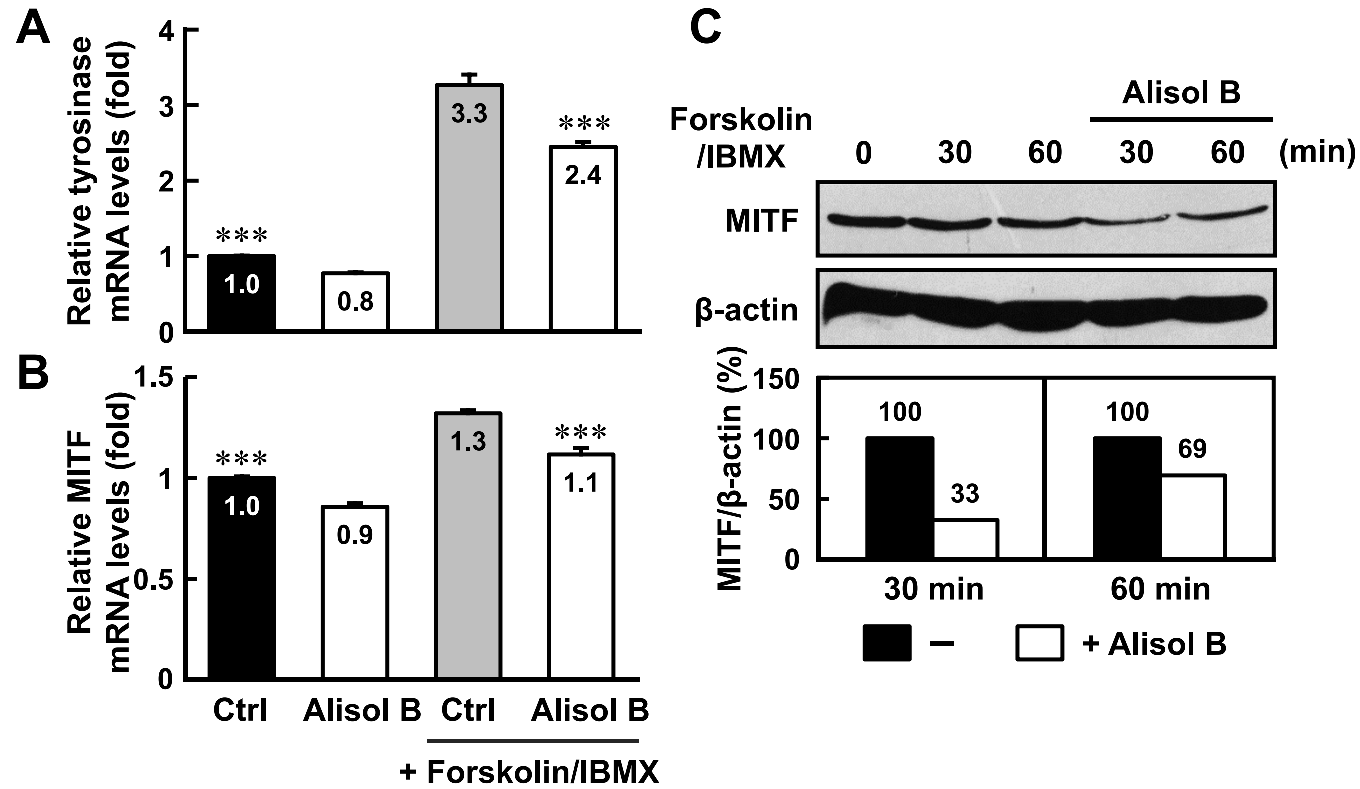

Fig.3. Yoshida et al. (2016)

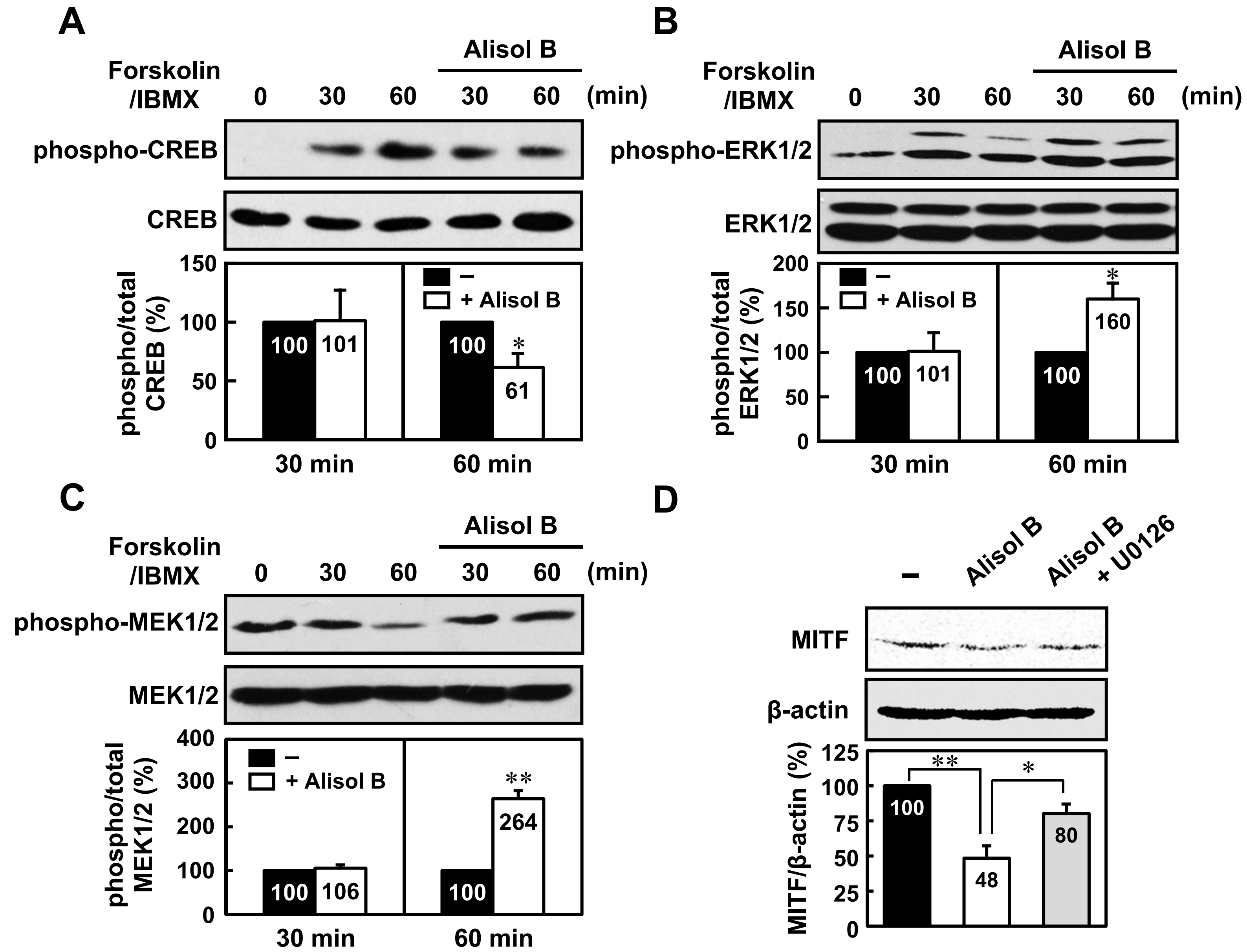

Fig.4. Yoshida et al. (2016)