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AVS-1357 inhibits melanogenesis via prolonged ERK activation

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In this study, we demonstrated that a derivative of imidazole, AVS-1357, is a novel skin-whitening compound. AVS-1357 was found to significantly inhibit melanin production in a dose-dependent manner; however, it did not directly inhibit tyrosinase. Furthermore, we found that AVS-1357 induced prolonged activation of extracellular signal-regulated kinase (ERK) and Akt, while it downregulated microphthalmia-associated transcription factor (MITF) and tyrosinase. It has been reported that the activation of ERK and/or Akt is involved in melanogenesis. Therefore, we examined the effects of AVS-1357 on melanogenesis in the absence or presence of PD98059 (a specific inhibitor of the ERK pathway) and/or LY294002 (a specific inhibitor of the Akt pathway). PD98059 dramatically increased melanogenesis, whereas LY294002 had no effect. Furthermore, PD98059 attenuated AVS-1357 induced ERK activation, as well as the downregulation of MITF and tyrosinase. These findings suggest that the effects of AVS-1357 occur via downregulation of MITF and tyrosinase, which is caused by AVS-1357-induced prolonged ERK activation. Taken together, our results indicate that AVS-1357 has the potential as a new skin whitening agent.

1. Introduction

Melanogenesis is dependent on the expression and activation of tyrosinase (Hearing and Tsukamoto 1991), which catalyzes the rate-limiting reaction of the melanogenic process (Hearing and Jimenez 1989; Slominski et al. 2004). Although treatment with potent tyrosinase inhibitors appears to be the best method of inhibiting melanogenesis, these inhibitors often have side effects such as skin irritation or permanent depigmentation. Additionally, some tyrosinase inhibitors require unacceptably high doses to produce visible effects. Therefore, the regulation of tyrosinase gene expression via signal transduction pathways is another option for the treatment of hyperpigmentary disorders.

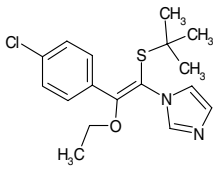
It is well known that the extracellular signal-regulated kinase (ERK) pathway is a major signaling cascade that regulates cell proliferation and differentiation in many types of cells (Cowley et al. 1994; Marshall 1995; Sale et al. 1995). In addition, it has been reported that inhibition of the ERK pathway in B16 melanoma cells induces cell differentiation and tyrosinase activity, thereby leading to hyperpigmentation (Englaro et al. 1998; Kim et al. 2003; Kim et al. 2002).

Abbreviations: CT, cholera toxin; MITF, microphthalmia-associated transcription factor; ERK, extracellular signal-regulated kinase; TPA, 12-O-tetradecanoylphorbol-13-acetate

We previously demonstrated that ERK activation by sphingosine-1-phosphate, ceramide, or sphingosylphosphorylcholine resulted in decreased melanogenesis via downregulation of MITF and tyrosinase (Kim et al. 2003, 2002, 2006). These findings suggest that the ERK pathway controls melanogenesis. However, it has also been reported that the Akt signaling pathway is involved in regulation of melanogenesis in G361 melanoma cells (Oka et al. 2000), and that specific inhibition of the Akt pathway by LY294002 stimulates melanin synthesis in mouse B16 melanoma cells (Busca et al. 1996; Khaled et al. 2002).

Microphthalmia-associated transcription factor (MITF) is a major transcription factor that regulates tyrosinase expression and melanin synthesis (Bertolotto et al. 1998; Lin and Fisher 2007; Yasumoto et al. 1997). Mutations in the MITF gene cause abnormal pigmentation of the skin and hair (Hughes et al. 1994; Tachibana 1997; Tassabehji et al. 1994). In addition, activation of the ERK pathway reportedly downregulates MITF levels, which leads to the inhibition of melanin synthesis (Englaro et al. 1998; Kim et al. 2003; Kim et al. 2002).

In the present study, we evaluated the effects of AVS-1357, a new derivative of imidazole, on melanin synthesis and tyrosinase activity in a spontaneously immortalized mouse melanocyte cell line (Mel-Ab). Furthermore, we examined the effects of AVS-1357 on the expression of MITF and tyrosinase to determine if they are regulated via the ERK and Akt pathways.



AVS-1357

2. Investigations and results

2.1. The cytotoxicity of AVS-1357 in Mel-Ab cells

The structure of AVS-1357 is shown above. While screening for new skin-whitening agents using Mel-Ab cells, we found that AVS-1357 exerted a strong hypopigmentary effect. Therefore, to determine if AVS-1357 can be safely used as a skin-whitening agent, we treated Mel-Ab cells with 0.01–50 μM AVS-1357 and then measured the cell viability using a crystal violet assay. As shown in Fig. 1, treatment with AVS-1357 at concentrations ranging from 0.1–20 μM had no cytotoxic effect on Mel-Ab cells. Accordingly, cells were exposed to 0.1–20 μM AVS-1357 for the remainder of the experiments.

2.2. AVS-1357 decreased tyrosinase activity and melanogenesis in Mel-Ab cells

To examine the hypopigmentation effect of AVS-1357, various concentrations of AVS-1357 were added to Mel-Ab cells, which were subsequently cultured for 4 days. The Mel-Ab cells were then photographed under a phase contrast microscope. AVS-1357 treatment was found to reduce melanin synthesis in a dose-dependent manner (Fig. 2A). In addition, measurement of the melanin content after 4 days of treatment revealed that AVS-1357 significantly inhibited melanogenesis in a dose-dependent manner (Fig. 2B). It is well-known that tyrosinase is a critical enzyme involved in the rate-limiting step of melanogenesis. Therefore, the tyrosinase activity was also measured following treatment with the same concentrations of AVS-1357. The results revealed that tyrosinase activity decreased in response to treatment with AVS-1357, and that these decreases occurred in a dose-dependant manner (Fig. 2C). The tyrosinase activity was also measured in a cell-free system using mushroom tyrosinase to determine if AVS-1357 inhibits the tyrosinase activity directly. However, AVS-1357 showed little inhibitory effect on tyrosinase activity (Fig. 2D). Taken together, these results indicate that the inhibitory effect of AVS-1357 on melanogenesis does not occur due to direct inhibition of tyrosinase.

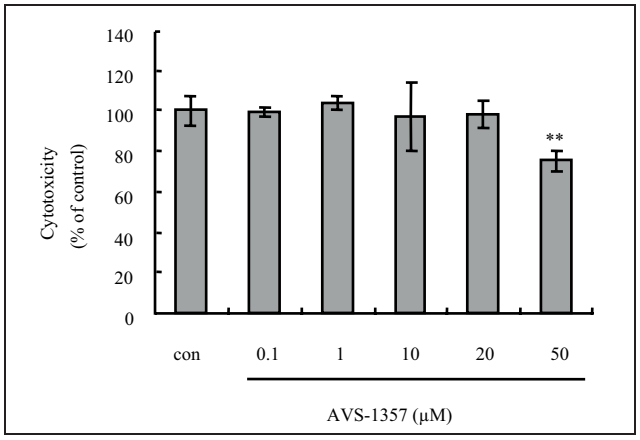


Fig. 1: Effects of AVS-1357 on Mel-Ab cell viability. Cells were serum-starved for 24 h and then treated with AVS-1357 at 0.1–50 μM for 24 h in serum-free media. The cell viabilities were then determined by crystal violet assays. Each determination was made in triplicate and the data shown are the means ± SD

2.3. AVS-1357 downregulated MITF and tyrosinase according to the activation of ERK and Akt

Because it has been reported that ERK and/or Akt activation is involved in the regulation of melanin synthesis (Hemesath et al. 1998; Khaled et al. 2002; Wu et al. 2000), we evaluated AVS-1357 to determine if it affects the ERK and Akt signaling pathways. As shown in Fig. 4, AVS-1357 activated ERK and Akt in a time dependent manner (Kim et al. 2003; Wu et al. 2000). It is interesting to note that this activation was sustained for at least 72 h. Next, we evaluated the reduced melanogenic protein expression to determine if it was responsible for the diminished melanin production. Although AVS-1357 did not directly inhibit tyrosinase activity in a cell free system, it did appear to decrease tyrosinase activity in Mel-Ab cells (Fig. 2C, D). Therefore, we evaluated the MITF and tyrosinase protein levels following AVS-1357 treatment. Treatment with AVS-1357 at a concentration of 10 μM reduced both the MITF and tyrosinase levels (Fig. 3), which was in accordance with its effects on the activation of ERK and Akt. Taken together, these findings suggest that the MITF and tyrosinase downregulation induced by AVS-1357 is related to its effects on the ERK and/or Akt pathway.

2.4. Effect of inhibition of the ERK and/or Akt pathway on melanin synthesis

To determine if AVS-1357 inhibits melanogenesis via the ERK and/or Akt pathway, Mel-Ab cells were cultured in the absence or presence of PD98059 (a specific inhibitor of the ERK pathway) and/or LY294002 (a specific inhibitor of the Akt pathway) for 4 days. The cells were then observed under a phase contrast microscope and photographed (Fig. 4A). PD98059 attenuated the AVS-1357 induced hypopigmentary effect, whereas LY294002 had no influence on this effect. In addition, PD98059 and LY294002 had no additive effects on attenuation of the AVS-1357 induced hypopigmentary effect. Melanin synthesis was also measured in the absence or presence of PD98059 and/or LY294002. Consistent with our microscopic observations, PD98059 blocked the hypopigmentary effects induced by AVS-1357, while LY294002 exerted no effects (Fig. 4B).

To verify the effects of PD98059 on the signaling pathways, we conducted the ERK activation experiment using PD98059. As shown in Fig. 5, treatment with AVS-1357 induced ERK activation as well as the downregulation of MITF and tyrosinase. However, treatment with PD98059 blocked the AVS-1357 induced ERK activation and led to recovery of the MITF and tyrosinase levels. Taken together, these results suggest that prolonged ERK activation contributes to the inhibition of melanin synthesis via decreased levels of MITF and tyrosinase.

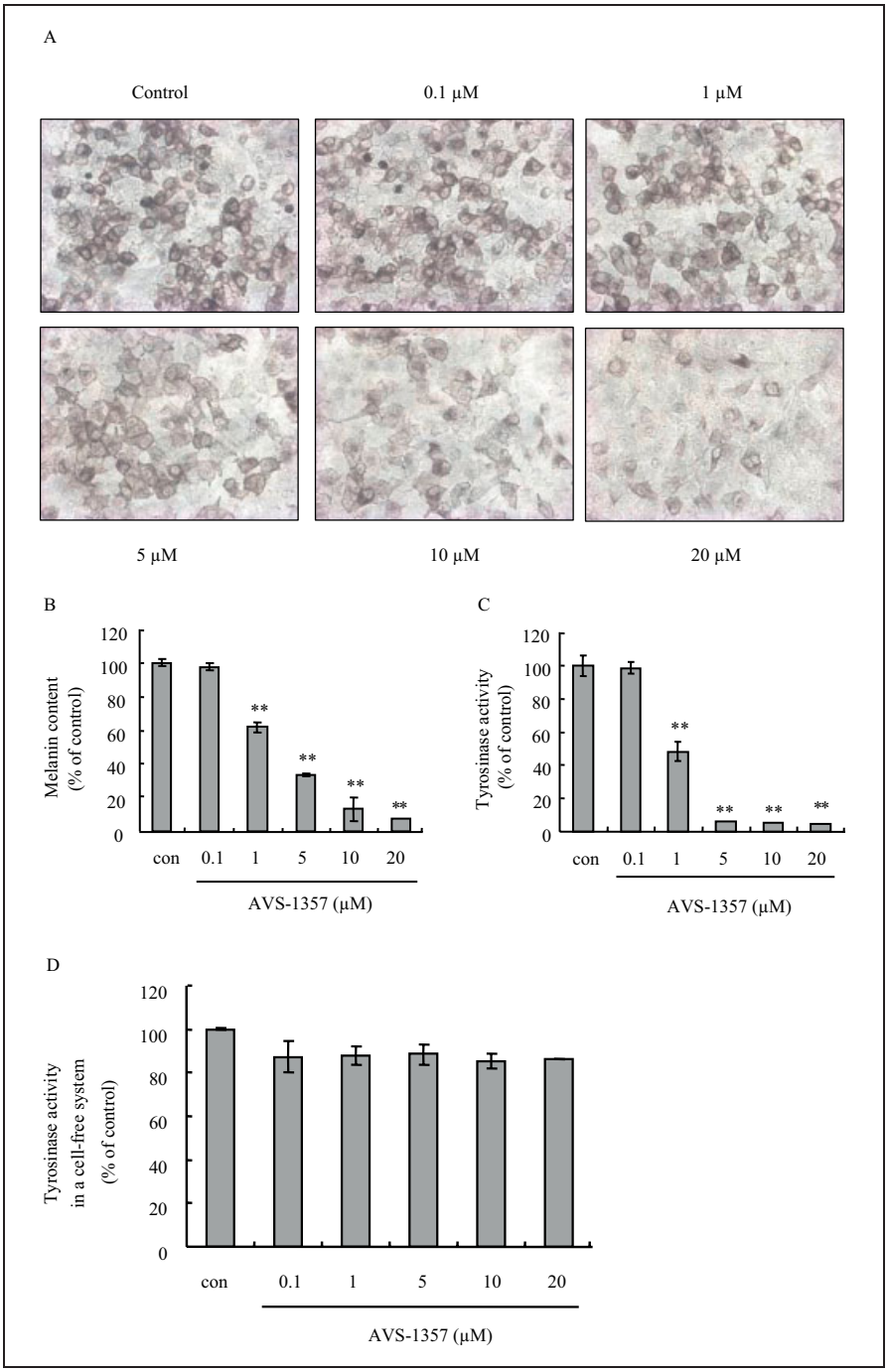


Fig. 2:
Effects of AVS-1357 on melanin synthesis and tyrosinase activity. Mel-Ab cells were cultured with 0.1–20 μM AVS-1357 for 4 days. (A) Phase contrast photomicrographs were then taken using a digital video camera, and (B) the melanin content, (C) tyrosinase activity, and (D) tyrosinase activity in a cell-free system were measured as described in the text. The results shown are the average of three replicate experiments ± SD. $^{**}p < 0.01$ when compared to an untreated control

3. Discussion

Hyperpigmentary skin diseases such as melasma, freckles, and solar lentigo result from increased melanin synthesis (Briganti et al. 2003). In addition, the dark skin also leads to many cosmetic problems. As a result, many studies have been conducted in attempts to develop new skin whitening agents. Because tyrosinase is a key enzyme involved in regulation of the rate-limiting step of melanin (Ando et al. 2007), many studies have focused on tyrosinase inhibitors using model enzymes such as mushroom and plant tyrosinases (Chang 2007; Kim and Uyama 2005). Although these inhibitors often show hypopigmenting effects *in vitro*, only a few substances have been found to have effective skin whitening activity in clinical trials. Nevertheless, we screened tyrosinase inhibitors for potential skin whitening activity.

In the present study, we evaluated AVS-1357 as a potential skin whitening compound. We found that, although AVS-1357 strongly inhibited melanogenesis, it did not inhibit tyrosinase directly. Furthermore, AVS-1357 was found to activate ERK for a prolonged length of time. It has been reported that ERK activation inhibits tyrosinase gene transcription (Englaro et al. 1998), which indicates that ERK activation leads to decreased tyrosinase levels and a subsequent reduction in melanin synthesis. Conversely, it has been reported that inhibition of the ERK pathway increases melanin production in human melanoma cells (Koo et al. 2002). Moreover, we have conducted several studies that have confirmed that activation of the ERK pathway results in decreased melanin production in human and mouse melanocytes (Kim et al. 2003, 2002, 2004; Park et al. 2004).

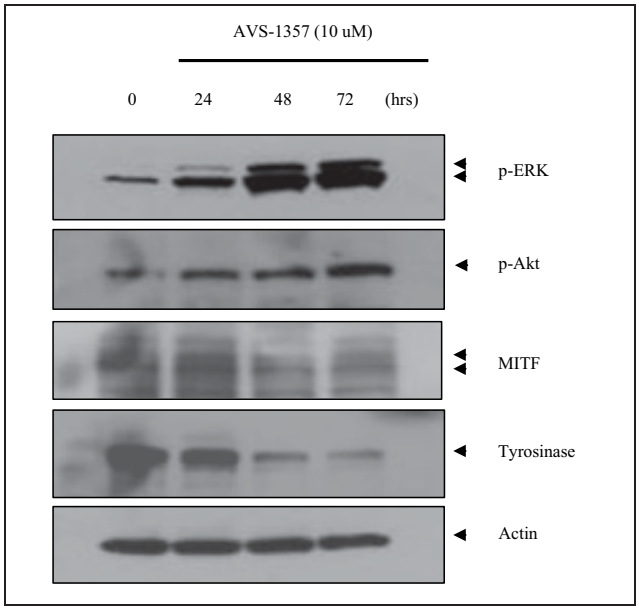


Fig. 3: Effects of AVS-1357 on ERK, Akt and melanogenic protein expression. Following 24 h of serum starvation, Mel-Ab cells were treated with 10 μM AVS-1357 for the indicated times. Whole cell lysates were then subjected to Western-blot analysis using antibodies against phospho-specific ERK (p-ERK), phospho-specific Akt (p-Akt), MITF, and tyrosinase. Equal protein loadings were confirmed using anti-actin antibody

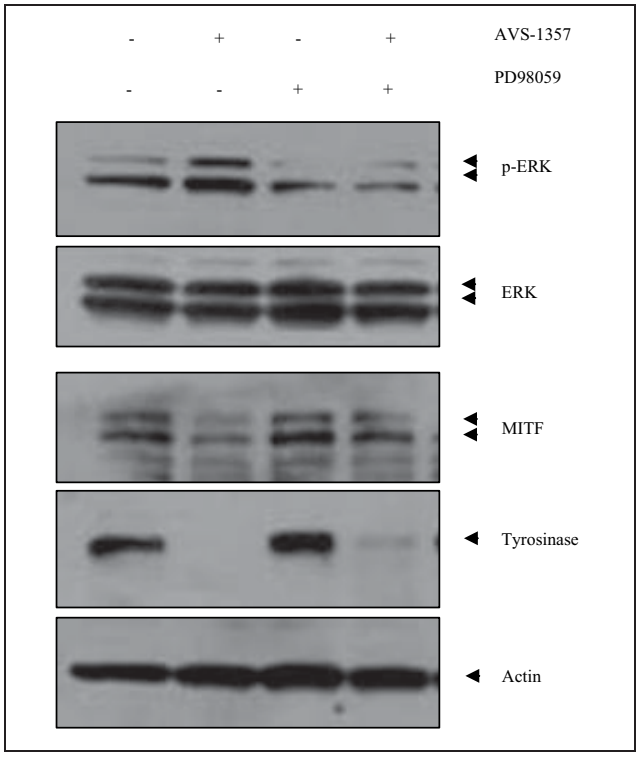


Fig. 5: Effects of inhibition of the ERK and/or Akt pathway on melanogenic protein expression. Following serum starvation, cells were cultured with 10 μM AVS-1357 for 48 h in the presence or absence of 20 μM PD98059. Whole cell lysates were then subjected to Western blot analysis using antibodies against phospho-specific ERK, MITF, or tyrosinase. Equal protein loading was confirmed using phosphorylation-independent ERK and actin antibodies

MITF is a major transcription factor that regulates the expression of melanogenic enzymes, including tyrosinase, TRP-1, and TRP-2 (Bentley et al. 1994; Busca and Ballotti 2000; Tachibana 2000). ERK activation is also known to induce MITF phosphorylation and its subsequent degradation, thereby reducing melanin synthesis (Hemesath et al. 1998; Wu et al. 2000; Xu et al. 2000). We also reported that sphingosine-1-phosphate immediately activates ERK and causes MITF degradation within 3 h of treatment (Kim et al. 2003). However, in the present study, the MITF and tyrosinase protein levels were unchanged after 6 h of treatment with AVS-1357 (data not shown). Furthermore, the MITF and tyrosinase protein levels were still unchanged after treatment with AVS-1357 for 24 h, although they did begin to decrease after treatment for

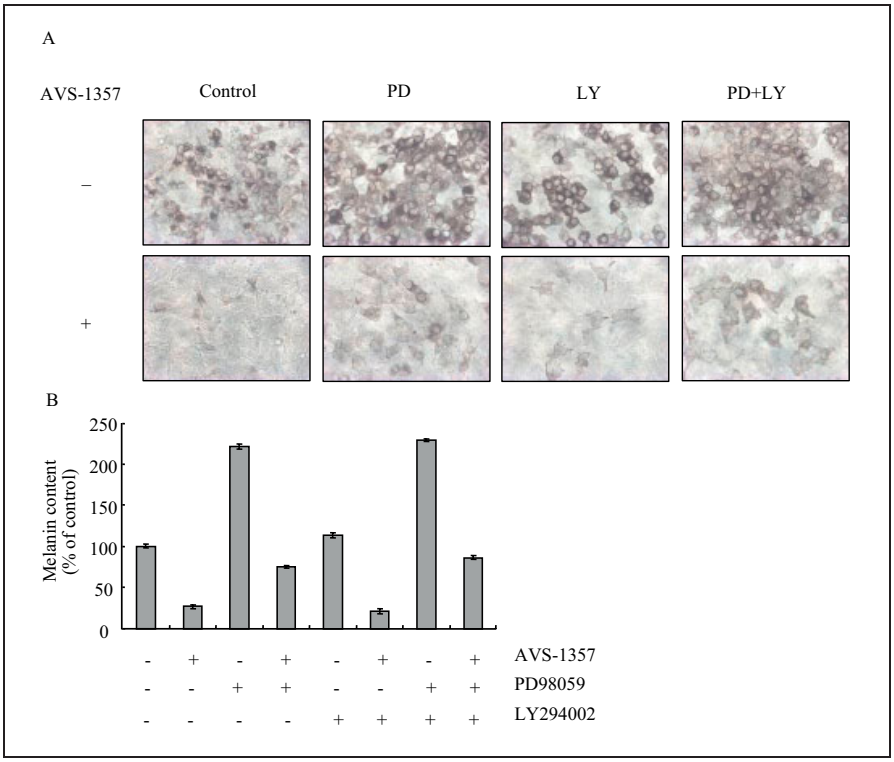


Fig. 4:
Effects of AVS-1357 on melanogenesis in the presence of PD98059 and/or LY294002. Mel-Ab cells were pretreated with 20 μM PD98059 for 1 h and/or 20 μM LY294002 for 10 min, after which they were cultured with 10 μM AVS-1357 for 4 days. (A) Phase contrast photographs were then taken using a digital video camera. (B) The melanin content was measured as described in the text. Each determination was made in triplicate and the data shown represent the mean ± SD

48 h. Conversely, ERK activation increased in a time dependent manner following treatment with AVS-1357. Although we previously suggested that MITF and subsequent tyrosinase downregulation is related to ERK activation (Kim et al. 2005), the mechanism by which ERK regulates MITF degradation and expression is not well understood. The results of this study suggest that short term activation of ERK may lead to MITF degradation, whereas prolonged activation of ERK may contribute to decreased MITF expression. Further studies should be conducted to elucidate the detailed mechanisms involved in ERK and MITF regulation.

In summary, the results of the present study demonstrate that AVS-1357 activates the ERK pathway in a prolonged manner. Furthermore, AVS-1357 was found to downregulate MITF and tyrosinase at the protein level, which contributed to the inhibition of melanin synthesis. Taken together, our results suggest that AVS-1357 has the potential as a skin-whitening agent.

4. Experimental

4.1. Materials

AVS-1357 (1-[(E)-1-tert-Butylsulfanyl-2-(4-chloro-phenyl)-2-ethoxy-vinyl]-1H-imidazole) was obtained from the Korea Research Institute of Chemical Technology. Cholera toxin (CT), 12-O-tetradecanoylphorbol-13-acetate (TPA), synthetic melanin, L-DOPA, and mushroom tyrosinase were obtained from Sigma (St. Louis, MO, USA).

4.2. Cell cultures

Mel-Ab is a mouse-derived spontaneously immortalized melanocyte cell line that produces large amounts of melanin (Dooley et al. 1994). Mel-Ab cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 100 nM TPA, 1 nM CT, 50 μg/ml streptomycin, and 50 U/ml penicillin at 37 °C under 5% CO_2.

4.3. Cell viability assay

Cell viability was determined using a crystal violet assay (Dooley et al. 1994). Briefly, cells were incubated in the presence of AVS-1357 (0.1–20 μM) for 24 h, after which the culture medium was removed and the cells were stained with 0.1% crystal violet in 10% ethanol for 5 min at room temperature. The cells were then rinsed four times with distilled water. Next, the crystal violet retained by the adherent cells was extracted with 95% ethanol, and the absorbance of the extract at 590 nm was then determined using an ELISA reader (TECAN, Salzburg, Austria).

4.4. Measurement of melanin contents and microscopy

The melanin content was measured using a slight modification of a previously described method (Tsuboi et al. 1998). Briefly, the cells were treated with various concentrations of AVS-1357 for 4 days. The cell pellets were then dissolved in 1 mL of 1 N NaOH at 100 °C for 30 min, after which they were centrifuged at 16,000g for 20 min. Next, the optical density (OD) of the supernatant was measured at 400 nm using an ELISA reader (TECAN). Prior to measuring the melanin content, the cells were observed under a phase contrast microscope (Olympus Optical Co., Tokyo, Japan) and photographed using the CoolSNAP$_{cf}$ digital video camera system (Roper Scientific, Inc., Tucson, AZ, USA), which was supported by the RS Image software (Roper Scientific).

4.5. Tyrosinase activity

The tyrosinase activity was assessed using a slight modification of a method that has been described elsewhere (Busca et al. 1996). Briefly, Mel-Ab cells were cultured in 6-well plates in the presence of various concentrations of AVS-1357 for 4 days. The cells were then washed with ice-cold PBS, after which they were lysed with 0.1 M phosphate buffer (pH 6.8) containing 1% Triton X-100. Next, the cells were disrupted by freezing and thawing, followed by clarification of the lysates by centrifugation at 10,000*g* for 5 min. The protein levels were then quantified and adjusted using lysis buffer, after which 90 μL of each lysate was placed in each well of a 96-well plate. Subsequently, 10 μL of 10 mM L-DOPA was added to each well. The control wells contained 90 μL of 0.1 M phosphate buffer (pH 6.8) and 10 μL of 10 mM L-DOPA. Following incubation at 37 °C, the absorbance at 475 nm was measured every 10 min for at least 1 h using an ELISA reader.

A cell-free assay system was used to examine the direct effects of AVS-1357 on tyrosinase activity. Briefly, 70 μL of phosphate buffer containing various concentrations of AVS-1357 were combined with 20 μL of 10 μg/mL mushroom tyrosinase and 10 μL of 10 mM L-DOPA and then added to each well. The plates were then incubated at 37 °C, after which the absorbance was measured at 475 nm.

4.6. Western blot analysis

Cells were lysed in cell lysis buffer [62.5 mM Tris-HCl (pH 6.8), 2% SDS, 5% β-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride, protease inhibitors (Complete^{TM}, Roche, Mannheim), 1 mM Na_3VO_4, 50 mM NaF, and 10 mM EDTA], after which 10 μg of protein per lane were separated by SDS-polyacrylamide gel electrophoresis. The separated proteins were then blotted onto PVDF membranes, which were subsequently saturated with 5% dried milk in Tris-buffered saline containing 0.4% Tween 20. Next, the blots were incubated with the appropriate primary antibodies at a dilution of 1 : 1000, and then further incubated with horseradish peroxidase-conjugated secondary antibody. The bound antibodies were detected using an enhanced chemiluminescence plus kit (Amersham International, Little Chalfont, UK).

4.7. Statistics

Differences among treatments were assessed by analysis of variance (ANOVA) followed by Dunnett's test. P values of <0.01 were taken to be significant.

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