Bioorganic & Medicinal Chemistry Letters 23 (2013) 4332–4335

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

The inhibitory effect of a synthetic compound, (*Z*)-5-(2,4-dihydroxybenzylidene) thiazolidine-2,4-dione (MHY498), on nitric oxide-induced melanogenesis

So Hee Kim [a], Yeon Ja Choi [a,b], Kyoung Mi Moon [a,b], Hye Jin Lee [c], Youngwoo Woo [c], Ki Wung Chung [a,b], Yuri Jung [a,b], Sora Kim [a,b], Pusoon Chun [d], Youngjoo Byun [e], Young Mi Ha [f], Hyung Ryong Moon [c,*], Hae Young Chung [a,b,*]

[a] *College of Pharmacy, Pusan National University, Geumjeong-Gu, Busan 609-735, Republic of Korea*
[b] *Molecular Inflammation Research Centre for Aging Intervention (MRCA), College of Pharmacy, Pusan National University, Geumjeong-Gu, Busan 609-735, Republic of Korea*
[c] *Laboratory of Medicinal Chemistry, College of Pharmacy, Pusan National University, Busan 609-735, Republic of Korea*
[d] *College of Pharmacy, Inje University, Gimhae, Gyeongnam 621-749, Republic of Korea*
[e] *College of Pharmacy, Korea University, Chungnam 339-700, Republic of Korea*
[f] *Department of Chemistry, Dong-A University, Hadan2-dong, Saha-gu, Busan 604-714, Republic of Korea*

ARTICLE INFO

Article history:
Received 9 March 2013
Revised 21 May 2013
Accepted 29 May 2013
Available online 6 June 2013

Keywords:
(*Z*)-5-(2,4-Dihydroxy benzylidene)thiazolidine-2,4-dione
Melanogenesis
Tyrosinase
Nitric oxide
Melanin
cGMP
MITF

ABSTRACT

Nitric oxide (NO) and the NO/PKG signaling pathway play crucial roles in ultraviolet (UV)-induced melanogenesis, which is known to be related to the induction of tyrosinase. In an attempt to find a novel anti-melanogenic agent, we synthesized (*Z*)-5-(2,4-dihydroxybenzylidene)thiazolidine-2,4-dione (MHY498). The purpose of this study was to investigate the effect of MHY498 on NO levels and on the NO-mediated signaling pathway using an *in vitro* model of melanogenesis. MHY498 inhibited 200 μM sodium nitroprusside (SNP, a NO donor)-induced NO generation, dose-dependently and suppressed tyrosinase activity and melanin synthesis induced by SNP in B16F10 melanoma cells. To investigate the effect of MHY498 on NO-mediated signaling pathway, guanosine cyclic 3′,5′-monophosphate (cGMP) activities were measured using a cGMP EIA Kit and western blotting was performed to determine the effects of MHY498 on the gene expressions of tyrosinase and microphthalmia-associated transcription factor (MITF). The increased activity of cGMP by SNP was reduced dose-dependently by pretreatment with MHY498. Furthermore, MHY498 suppressed the expressions of tyrosinase and MITF stimulated by SNP. This study shows that enhancement of tyrosinase gene expression *via* the cGMP pathway is a probable primary mechanism of NO-induced melanogenesis and that the NO-mediated signaling pathway with the expression of MITF enhances melanogenesis. In addition, MHY498 was found to scavenge NO and to suppress the activity of the NO-mediated signaling pathway, and thus, to subsequently down-regulate tyrosinase expression and melanogenesis. This study suggests that MHY498 is a promising anti-melanogenic agent that targets the NO-induced cGMP signaling pathway.

Ultraviolet (UV) radiation-mediated melanogenic response in melanocytes and melanoma cells is a complex process that remains to be elucidated. It has been reported that several melanogenic factors are released by keratinocytes and other cells surrounding melanocytes in the skin following exposure to UV. These melanogenic factors include nitric oxide (NO), adrenocorticotropic hormone (ACTH), α-melanocyte stimulating hormone (α-MSH),[1,2] endothelin-1 (ET-1),[3,4] prostaglandins,[5] thymidine dinucleotide,[6] and histamine.[7,8]

In the mid-1990s, Roméro-Graillet et al. found that the effect of UV on human melanocyte cultures was similar to that of exogenous NO or an analog of guanosine cyclic 3′,5′-monophosphate

Abbreviations: ACTH, adrenocorticotropic hormone; cAMP, adenosine 3′,5′-cyclic monophosphate; ANOVA, analysis of variance; CREB, adenosine 3′,5′-cyclic monophosphate responsive element binding protein; pCREB, phosphorylated form of adenosine 3′,5′-cyclic monophosphate responsive element binding protein; L-DOPA, L-3,4-dihydroxyphenylalanine; ET-1, endothelin-1; EtOH, ethanol; GC, guanylate cyclase; cGMP, guanosine cyclic 3′,5′-monophosphate; MITF, microphthalmia-associated transcription factor; α-MSH, alpha-melanocyte stimulating hormone; L-NAME, *N*-nitro-L-arginine methyl ester hydrochloride; NO, nitric oxide; PKA, protein kinase A; PKG, protein kinase G; SDS, sodium dodecyl sulphate; SNP, sodium nitroprusside; UV, ultraviolet; UVA, ultraviolet A.

* Corresponding authors. Tel.: +82 51 510 2815; fax: +82 513 6754 (H.R.M.); tel.: +82 51 510 2814; fax: +82 518 2821 (H.Y.C.).
E-mail addresses: mhr108@pusan.ac.kr (H.R. Moon), hyjung@pusan.ac.kr (H.Y. Chung).

0960-894X/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.bmcl.2013.05.094

(cGMP), and that melanogenesis was blocked by inhibitors of cGMP-dependent kinase.[9] It was also demonstrated that UV-irradiated keratinocytes released sufficient NO to induce melanogenesis in keratinocyte/melanocyte co-cultures.[10] Furthermore, in vivo experiments on guinea pigs have shown that the topical application of *N*-nitro-L-arginine methyl ester hydrochloride (L-NAME, a NO synthase inhibitor) inhibits UV-induced melanogenesis and reduces melanin content and the number of histochemically DOPA-positive melanocytes.[11] Thus, these results suggested that NO played a crucial role in the induction of melanogenesis.

NO directly interacts with and activates heme-containing proteins, like guanylate cyclase (GC), which induces the production of cGMP, and consequently, activates protein kinase G (PKG).[12,13] It has also been reported that NO and the cGMP signaling pathway stimulate the gene expression of tyrosinase.[9,14] Tyrosinase is the most well-characterized enzyme which catalyzes the two initial rate-limiting steps of melanogenesis, that is, the hydroxylation of L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) and the oxidation of L-DOPA to DOPAquinone.[15,16] Because of its key role in melanogenesis, tyrosinase continues to be an attractive target for research on the biochemistry of melanogenesis and on the management of hyperpigmentation disorders.

We synthesized (*Z*)-5-(2,4-dihydroxybenzylidene)thiazolidine-2,4-dione (MHY498) in the following manner. Piperidine (0.03 mL, 0.3 mmol) was added to a stirred solution of substituted 2,4-dihydroxybenzaldehyde (200 mg, 1.45 mmol) and thiazolidine-2,4-dione (204 mg, 1.74 mmol) in EtOH (4 mL) and the reaction mixture was refluxed for 24 h (Fig. 1). During reflux, precipitates were formed, and after cooling, water was added and the precipitates were filtered off using a Buchner funnel. The filter cake so obtained was washed with water/ethanol (5:1), and then subjected to flash silica gel column chromatography using chloroform/methanol (10:1) as eluent to give MHY498 (156.1 mg, 45.4%) as a solid. MHY498 was dissolved in DMSO as a vehicle, and same volume of DMSO was added to the untreated control groups and the groups treated with only SNP.

In the present study, we focused on the inhibitory effect of MHY498 on NO-induced signaling pathway leading anti-melanogenesis in B16F10 melanoma cells.

NO-scavenging activity of MHY498 was examined in B16F10 melanoma cells. In these cells, MHY498 pretreatment scavenged NO induced by 200 μM sodium nitroprusside (SNP, a NO donor) (Fig. 2). The percentage of NO levels in cells treated with MHY498 was 200.00 ± 10.46% at 0.5 μM, 182.74 ± 11.69% at 4 μM, and 172.14 ± 3.89% at 16 μM, as compared with 237.84 ± 4.12% in cells treated with SNP alone and 205.82 ± 8.52% in cells treated with 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO) 16 μM, a well-known NO scavenger. Furthermore, the NO-scavenging activity of MHY498 was dose-dependent and more potent than that of carboxy-PTIO.

To investigate the effect of MHY498 on NO-induced cGMP production, intracellular cGMP levels were measured in the presence or absence of MHY498. As shown in Figure 3, intracellular cGMP levels were increased by SNP and these increases were decreased

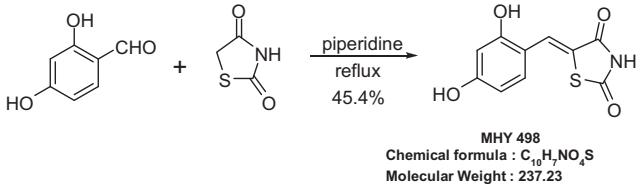

Figure 1. Brief scheme of the synthesis of (*Z*)-5-(2,4-dihydroxybenzylidene) thiazolidine-2,4-dione (MHY498).

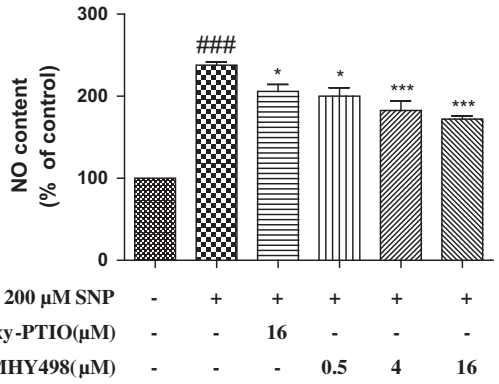

Figure 2. NO scavenging activity of MHY498 in B16F10 melanoma cells. The measurement of NO levels was followed by measuring the accumulation of nitrite in conditioned medium using the Griess assay. B16F10 melanoma cells were treated with various concentrations of MHY498 (0.5, 4, 16 μM) or 16 μM carboxy-PTIO for 1 h. 200 μM SNP was then added and cells were incubated for 48 h. The result is expressed as a percentage of the control, and each data is expressed as mean ± SE ($n = 6$). Statistical results were obtained using one-factor ANOVA: $^{\#\#\#}p$ <0.001 versus untreated control group, $^{***}p$ <0.001, $^{*}p$ <0.05 versus the group treated with only SNP, respectively.

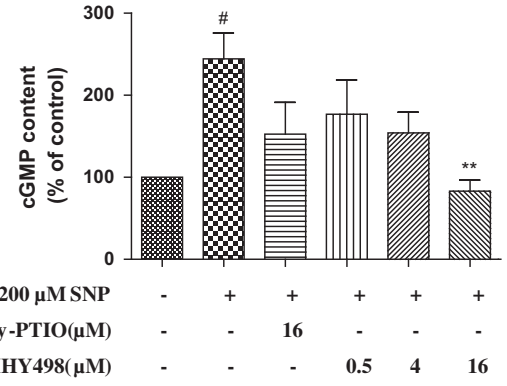

Figure 3. The effect of MHY498 on cGMP content in B16F10 melanoma cells. cGMP levels were measured using a Cyclic GMP EIA Kit (Cayman Chemical Company). B16F10 melanoma cells were treated with various concentrations of MHY498 (0.5, 4, 16 μM) or 16 μM carboxy-PTIO. After 1 h, 200 μM SNP was then added and cells were incubated for 30 min. cGMP levels were determined under non-acetylation conditions according to the manufacturer's instructions. The result is expressed as a percentage of the control, and each data is expressed as mean ± SE ($n = 4$). Statistical results were obtained using one-factor ANOVA: $^{\#}p$ <0.05 versus untreated control group, $^{**}p$ <0.01 versus the group treated with only SNP, respectively.

dose-dependently by MHY498. The percentage levels of cGMP in cells treated with MHY498 were 176.86 ± 41.84% at 0.5 μM, 154.01 ± 25.43% at 4 μM, and 83.08 ± 13.67% at 16 μM, as compared with untreated controls. Furthermore, the suppressive effect of 16 μM MHY498 on cGMP production was more potent than that of 16 μM carboxy-PTIO (83.08 ± 13.67% *vs* 152.61 ± 38.76%). We believe that these decreased levels of cGMP probably suppress PKG activity.

As previously mentioned, it has been reported that NO and the cGMP signaling pathway stimulate the gene expression of tyrosinase.[9,14] Furthermore, it is known that microphthalmia-associated transcription factor (MITF) is a transcription factor responsible for the expression of tyrosinase.[17] To elucidate the mechanism whereby MHY498 reduces NO-induced melanogenesis in B16F10 melanoma cells, we measured tyrosinase and MITF protein levels by Western blot analysis. As shown in Figure 4, the protein expression levels of tyrosinase and MITF were increased by 200 μM SNP and reduced dose-dependently by MHY498. These results imply that

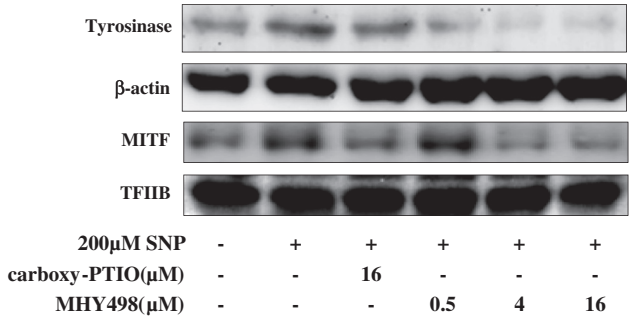

Figure 4. The effect of MHY498 on the expressions of tyrosinase and nuclear MITF. Protein levels were measured by Western blotting. Samples were resolved on 10% SDS–PAGE gels and transferred to nitrocellulose membranes. β-Actin and TFIIB blots were used to ensure equivalent protein loading in cytosolic and nuclear fractions, respectively.

the decrease in tyrosinase protein levels in B16F10 melanoma cells treated with MHY498 can be attributed to blocking of the cGMP-dependent signaling pathway, which is known to be involved in the expression of MITF.

It has also been reported that NO facilitates the phosphorylation of adenosine 3′,5′-cyclic monophosphate (cAMP) response element binding protein (CREB), which acts as an important transcription factor in melanogenesis.[18] Therefore, we measured the phosphorylation levels of CREB in NO-treated B16F10 melanoma cells by Western blotting. The phosphorylation levels of CREB were increased by SNP, but decreased by carboxy-PTIO and by MHY498, respectively, at 16 μM. However, at MHY498 concentrations of 0.5 and 4 μM, the phosphorylation levels of CREB were not substantially changed (Fig. 5). Any significant differences have not been found in the total CREB levels. These results indicate that MHY498 affects the phosphorylation of CREB not reducing total CREB levels. Therefore, we considered that NO-induced tyrosinase expression was mediated *via* the cGMP/CREB/MITF signaling pathway leading to NO-stimulated melanogenesis, and that this process was blocked by the scavenging of intracellular NO by MHY498.

The cAMP/PKA/CREB pathway stimulated by paracrine α-MSH is the major melanogenic signal transduction in melanocytes, and results in the upregulation of tyrosinase.[19] However, it is also known that the NO-induced cGMP/PKG signaling pathway is also important in melanogenesis. Furthermore, it has been reported that cGMP inhibited cAMP phosphodiesterase, and that this led to an increase in the level of cAMP,[20] a strong stimulator of melanogenesis.[21] Thus, cGMP induces the cAMP/PKA signaling pathway as well as PKG signaling pathway, and both signaling pathways could be correlated in melanogenesis.

Next, we examined the inhibitory effect of MHY498 on tyrosinase activity in B16F10 melanoma cells treated with SNP. As shown in Figure 6, SNP stimulated tyrosinase activity in B16F10 melanoma cells, and MHY498 pretreatment inhibited this effect

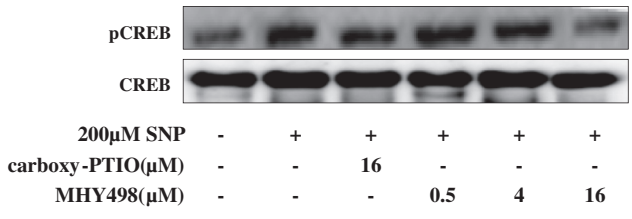

Figure 5. The effect of MHY498 on the phosphorylation of CREB. Protein levels were measured by Western blotting. The samples were resolved on 10% SDS–PAGE gels and transferred to nitrocellulose membranes. pCREB is the phosphorylated form of CREB.

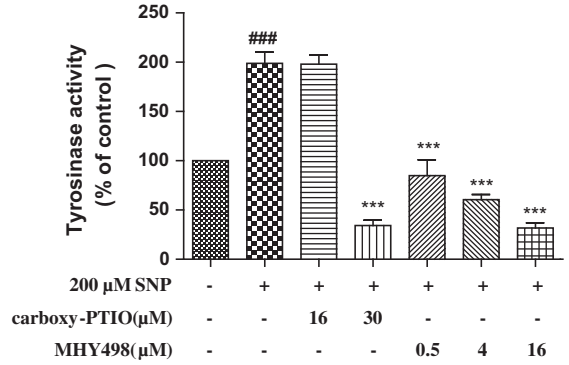

Figure 6. Inhibitory effect of MHY498 on tyrosinase activity in B16F10 melanoma cells in the presence of 200 μM SNP. B16F10 melanoma cells were treated with various concentrations of MHY498 (0.5, 4, 16 μM) or carboxy-PTIO (16, 30 μM). After 1 h, 200 μM SNP was added and then cells were incubated for 48 h. Tyrosinase activities were measured using the method described in our previous study.[17] The result is expressed as a percentage of the control, and each data is expressed as mean ± SE ($n = 5$). Statistical results were obtained using one-factor ANOVA: $^{###}p$ <0.001 versus untreated control group, $^{***}p$ <0.001 versus the group treated with only SNP, respectively.

of SNP. Tyrosinase activities in cells treated with MHY498 were 84.85 ± 16.12% at 0.5 μM, 60.47 ± 5.31% at 4 μM, and 31.79 ± 5.23% at 16 μM as compared with untreated controls, whereas in cell treated with SNP alone it was 198.83 ± 11.46%. Tyrosinase activities of carboxy-PTIO were 197.98 ± 9.23% at 16 μM and 34.33 ± 5.41% at 30 μM. These results indicate that MHY498 more potently inhibits tyrosinase activity induced by NO than carboxy-PTIO.

The inhibitory effect of MHY498 reduced cellular melanin contents in B16F10 melanoma cells (Fig. 7), whereas SNP increased melanin contents. Furthermore, the melanin contents induced by SNP were dose-dependently reduced by MHY498 pretreatment. The percentages of melanin in cells treated with MHY498 were; 151.43 ± 30.09% at 0.5 μM, 114.36 ± 4.60% at 4 μM, and 100.29 ± 6.26% at 16 μM as compared with 282.59 ± 47.06% in cells treated with SNP alone. At concentrations of 16 μM, the percentages of melanin content in cells treated with MHY498 and carboxy-PTIO were 100.29 ± 6.26% and 173.24 ± 10.99%, respectively.

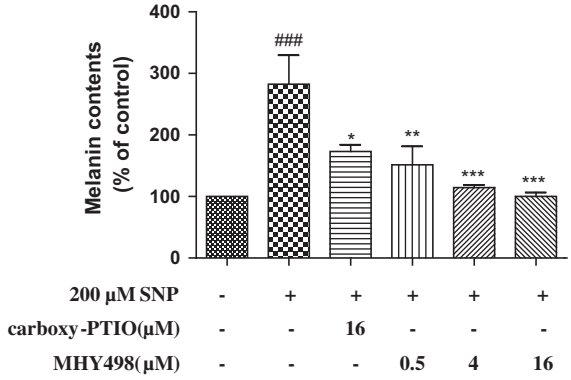

Figure 7. The effect of MHY498 on the melanin contents of B16F10 melanoma cells in the presence of 200 μM SNP. B16F10 melanoma cells were treated with various concentrations of MHY498 (0.5, 4, 16 μM) or 16 μM carboxy-PTIO. After 1 h, 200 μM SNP was then added and cells were incubated for 48 h. Melanin contents were determined using a modification of the method described by Bilodeau et al.[26] The result is expressed as a percentage of the control, and each data is expressed as mean ± SE ($n = 6$). Statistical results were obtained by one-factor ANOVA: $^{###}p$ <0.001 versus untreated control group, $^{***}p$ <0.001, $^{**}p$ <0.01, $^{*}p$ <0.05 versus the group treated with only SNP, respectively.

These results suggest that MHY498 is a more potent inhibitor of melanogenesis than carboxy-PTIO.

Although the role of NO in melanogenesis could be significant, there are limited pharmacological studies that investigate new compounds targeting NO-induced melanogenesis. In one study, ascorbic acid was found to modulate UVA-induced melanogenesis, which is partially related to the NO system.[22] In others, pycnogenol, a natural extract from pine bark, and proanthocyanidins found in persimmon peel partially suppressed melanin biosynthesis *via* NO and $ONOO^-$ scavenging.[23,24] In a previous study conducted in our laboratory, MHY966 (2-bromo-4-(5-chlorobenzo[*d*]thiazol-2-yl)phenol), a new synthetic compound, was also found to have an inhibitory effect of NO-induced melanogenesis.[25]

In summary, we found that MHY498 has NO scavenging activity and inhibits cGMP production induced by SNP, a NO donor. Furthermore, MHY498 inhibited the NO-induced gene expressions of tyrosinase and nuclear MITF, and the phosphorylation of CREB, which was followed by reductions in tyrosinase activity and cellular melanin contents. These findings showed that MHY498 suppressed NO production and the NO-mediated signaling pathway, which lead to inhibit melanogenesis. Although further *in vivo* study is needed, our results indicate that MHY498 is a promising candidate of pharmacological and cosmetic agent for the treatment of skin hyperpigmentation.

Acknowledgments

This work was supported by a National Research Foundation of Korea (NRF) grant funded by the Korean government (MEST) (No. 2009-0083538). We thank the Aging Tissue Bank for providing us with research materials.

References and notes

1. Schauer, E.; Trautinger, F.; Kock, A.; Schwarz, A.; Bhardwaj, R.; Simon, M.; Ansel, J. C.; Schwarz, T.; Luger, T. A. *J. Clin. Invest.* **1994**, *93*, 2258.
2. Thody, A. J.; Graham, A. *Pigment Cell Res.* **1998**, *11*, 265.
3. Imokawa, G.; Miyagishi, M.; Yada, Y. *J. Invest. Dermatol.* **1995**, *105*, 32.
4. Imokawa, G.; Kobayashi, T.; Miyagishi, M.; Higashi, K.; Yada, Y. *Pigment Cell Res.* **1997**, *10*, 218.
5. Nordlund, J. J.; Collins, C. E.; Rheins, L. A. *J. Invest. Dermatol.* **1986**, *86*, 433.
6. Eller, M. S.; Yaar, M.; Gilchrest, B. A. *Nature* **1994**, *372*, 413.
7. Yoshida, M.; Takahashi, Y.; Inoue, S. *J. Invest. Dermatol.* **2000**, *114*, 334.
8. Slominski, A.; Tobin, D. J.; Shibahara, S. Wortsman *Physiol. Rev.* **2004**, *84*, 1155.
9. Romero-Graillet, C.; Aberdam, E.; Biagoli, N.; Massabni, W.; Ortonne, J. P.; Ballotti, R. *J. Biol. Chem.* **1996**, *271*, 28052.
10. Romero-Graillet, C.; Aberdam, E.; Clement, M.; Ortonne, J. P.; Ballotti, R. *J. Clin. Invest.* **1997**, *99*, 635.
11. Horikoshi, T.; Nakahara, M.; Kaminaga, H.; Sasaki, M.; Uchiwa, H.; Miyachi, Y. *Pigment Cell Res.* **2000**, *13*, 358.
12. Murad, F. *Adv. Pharmacol.* **1994**, *26*, 19.
13. McDonald, L. J.; Murad, F. *Proc. Soc. Exp. Biol. Med.* **1996**, *211*, 1.
14. Sasaki, M.; Horikoshi, T.; Uchiwa, H.; Miyachi, Y. *Pigment Cell Res.* **2000**, *13*, 248.
15. Busca, R.; Ballotti, R. *Pigment Cell Res.* **2000**, *13*, 60.
16. Ebanks, J. P.; Wickett, R. R.; Boissy, R. E. *Int. J. Mol. Sci.* **2009**, *10*, 4066.
17. Han, Y. K.; Park, Y. J.; Ha, Y. M.; Park, D.; Lee, J. Y.; Lee, N.; Yoon, J. H.; Moon, H. R.; Chung, H. Y. *Biochim. Biophys. Acta* **2012**, *1820*, 542.
18. Galeotti, N.; Ghelardini, C. *Psychopharmacology* **2013**, *227*, 149.
19. Horibe I, Satoh Y, Shiota Y, Kumagai A, Horike N, Takemori H, Uesato S, Sugie S, Obata K, Kawahara H, Nagaoka Y. *J. Nat. Med.* 2012, [Epub ahead of print].
20. Brechler, V.; Pavoine, C.; Hanf, R.; Garbarz, E.; Fischmeister, R.; Pecker, F. *J. Biol. Chem.* **1992**, *267*, 15496.
21. Abdel-Malek, Z.; Swope, V. B.; Suzuki, I.; Akcali, C.; Harriger, M. D.; Boyce, S. T.; Urabe, K.; Hearing, V. J. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 1789.
22. Panich, U.; Tangsupa-a-nan, V.; Onkoksoong, T.; Kongtaphan, K.; Kasetsinsombat, K.; Akarasereenont, P.; Wongkajornsilp, A. *Arch. Pharm. Res.* **2011**, *34*, 811.
23. Kim, Y. J.; Yokozawa, T. *Biol. Pharm. Bull.* **2009**, *32*, 1155.
24. Kim, Y. J.; Kang, K. S.; Yokozawa, T. *Food Chem. Toxicol.* **2008**, *46*, 2466.
25. Choi, Y. J.; Uehara, Y.; Park, J. Y.; Chung, K. W.; Ha, Y. M.; Kim, J. M.; Song, Y. M.; Chun, P.; Park, J. W.; Moon, H. R.; Chung, H. Y. *J. Dermatol. Sci.* **2012**, *68*, 164.
26. Bilodeau, M. L.; Greulich, J. D.; Hullinger, R. L.; Bertolotto, C.; Ballotti, R.; Andrisani, O. M. *Pigment Cell Res.* **2001**, *14*, 328.