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Induction of pigmentation by a small molecule tyrosine kinase inhibitor nilotinib

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ABSTRACT

Skin color is determined by the melanin pigments that are produced in melanocytes then transferred to surrounding keratinocytes. Despite the growing number of commercial products claiming the pigmentation-regulatory effects, there is still a demand for the development of new materials that are safe and more efficacious. We tried to screen the pigmentation-regulatory materials using a commercially available drugs, and found that nilotinib could induce pigmentation in melanoma cells. When HM3KO melanoma cells were treated with nilotinib, melanin content was increased together with increase of tyrosinase activity. Nilotinib increased the expression of pigmentation-related genes such as MITF, tyrosinase and TRP1. Consistent with these results, the protein level for MITF, tyrosinase, and TRP1 was significantly increased by nilotinib. To delineate the action mechanism of nilotinib, we investigated the effects of nilotinib on intracellular signaling. As a result, nilotinib decreased the phosphorylation of AKT, while increased the phosphorylation of CREB. The pretreatment of PKA inhibitor H89 markedly blocked the nilotinib-induced phosphorylation of CREB. In accordance with, pretreatment of H89 significantly inhibited the nilotinib-induced pigmentation, indicating that nilotinib induces pigmentation via the activation of PKA signaling. Together, our data suggest that nilotinib can be developed for the treatment of hypopigmentary disorder such as vitiligo.

1. Introduction

Melanocytes are responsible for skin color by making pigmentary substances called melanins. After being synthesized in melanocytes, melanin pigments are transferred to surrounding keratinocytes and then locate above the nucleus of keratinocytes [1,2]. Because that skin keratinocytes is directly and continuously exposed to ultraviolet (UV) light, it is believed that the primary role of melanocytes is the protection of epidermal cells by making melanin pigments that have intrinsic property of scavenging the UV-induced free radical species [3].

It is well described that UV irradiation provokes mutagenic and cytotoxic DNA damages [4]. Consequently, the DNA damages activate many of cellular events such as p53-dependent gene expression of which targets include proopiomelanocortin (POMC) in keratinocytes [5]. The POMC gene product is further processed by proteases into several small peptide ligands such as adrenocorticotropic hormone (ACTH) and α-melanocyte stimulating hormone (α-MSH). An important melanogenic inducer α-MSH then binds to melanocortin 1 receptor (MC1R) on melanocytes, which triggers the activation of cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA) signaling pathway. As a result, the expression of pigmentation-related genes including microphthalmia-associated transcription factor (MITF), tyrosinase, and tyrosinase-related proteins (TRPs) is increased in melanocytes [6,7].

The development of pigmentation-regulatory materials has long acquired a great interest in both the dermatological and cosmetical

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viewpoints. For example, pigmentation-inducing materials is required for the treatment of skin disease such as vitiligo. In contrast, there is a great demand for development of skin-lightening agents in the beauty industry. To develop the regulatory drugs on pigmentation, many investigators are performing massive screening and validation tests using various candidate materials obtained from a variety sources [8–10]. We performed screening test using the compounds commercially available, and found that nilotinib, a small molecule tyrosine kinase inhibitor, had the pigmentation-inducing potential in melanoma cells. In this study, we provide the evidence that nilotinib induces pigmentation via the activation of PKA signaling.

2. Materials and methods

2.1. Cell culture

The human melanoma cell line HM3KO was maintained in Minimum Essential Medium (MEM), supplemented with 10% fetal bovine serum (FBS) and antibiotics (Life Technologies Corporation, Grand Island, NY). The compound nilotinib was purchased from Enzo Life Sciences Inc. (Farmingdale, NY) and dissolved in dimethyl sulfoxide (DMSO) and then diluted with culture medium (final concentrations of DMSO is 0.1%). The PKA inhibitor H89 was purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Cytotoxicity test

HM3KO melanoma cells were treated with nilotinib for 24 h, then medium was replaced with fresh medium containing 0.5 mg/ml 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) solution. Cells were incubated for an additional 4 h, and then formazan crystal was dissolved with DMSO. Cell viability was determined by measuring optical density at 570 nm using an ELISA reader.

2.3. Melanin content and tyrosinase activity

For determination of pigmentation, cells were collected and pelleted by centrifugation. Melanin pigment was dissolved in 1 N NaOH at 100 °C for 30 min, and quantified by measuring optical density at 405 nm. For determination of tyrosinase activity, cells were lysed in Pro-Prep protein extraction solution (Intron, Daejeon, Korea), then lysate was clarified by centrifugation. After quantification, 250 μg of total protein in 100 μl of lysis buffer was transferred into the 96-well plate, and 100 μl of 1 mM L-DOPA was added. After incubation for 30 min at 37 °C, absorbance was measured at 405 nm.

2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNAs were isolated using Easy-blue RNA extraction kit (Intron, Daejeon, Korea). Two μg of total RNAs were reverse transcribed with moloney-murine leukaemia virus (M-MLV) reverse transcriptase (RTase) (Elpis Biotech, Daejeon, Korea). Aliquots of RT mixture were subjected to PCR cycles with appropriate primer sets. The sequences for primers were as follows: MITF, 5′-ACCTTCTCTTTGCCAGTCCA-3′ and 5′-CGGATATAGTCCACGGATCG-3'; tyrosinase, 5′-AGGCAGAGGTTCCTGTCAGA-3′ and 5′-CTATGC-CAAGGCAGAAAAGC-3'; TRP1, 5′-CTCCTGCACACCTTCACAGA-3′ and 5′-TCAGTGAGGAGAGGCTGGTT-3'; GAPDH, 5′-CGACCACTTTGT-CAAGCTCA-3′ and 5′-AGGGGTCTACATGGCAACTG-3'.

2.5. Western blot analysis

Cells were harvested by centrifugation and then lysed in Pro-Prep protein extraction solution (Intron, Daejeon, Korea). After vigorous pipetting, extracts were centrifuged for 15 min at 15,000 rpm. Total protein was measured using a BCA protein assay kit (Thermo Scientific, Rockford, IL). Samples (20–30 μg protein per lane) were run on SDS-polyacrylamide gels, transferred onto nitrocellulose membranes and incubated with appropriate antibodies for overnight at 4 °C with gentle agitation. Blots were then incubated with peroxidase-conjugated secondary antibodies for 30 min at room temperature, and visualized by enhanced chemiluminescence (Intron, Daejeon, Korea). The following primary antibodies were used in this study: MITF, tyrosinase, TRP1 (Santa Cruz Biotechnologies, Santa Cruz, CA); Actin (Sigma-Aldrich, St. Louis, MO); phospho-AKT, AKT, phospho-CREB, CREB (Cell Signaling Technology, Danvers, MA).

2.6. Statistical analysis

All data were derived from at least three independent experiments. The results were presented as a percentage to control ± standard deviation (SD). Data were evaluated statistically by one-way ANOVA or Student's t-test using SPSS software v 22.0 (IBM, Seoul, Korea). Statistical significance was set at $p < 0.01$.

3. Results

Nilotinib was originally developed as a small molecule inhibitor for BCR-ABL tyrosine kinase (Fig. 1A) [11]. We first checked the cytotoxic effect of nilotinib on HM3KO melanoma cells. As a result,

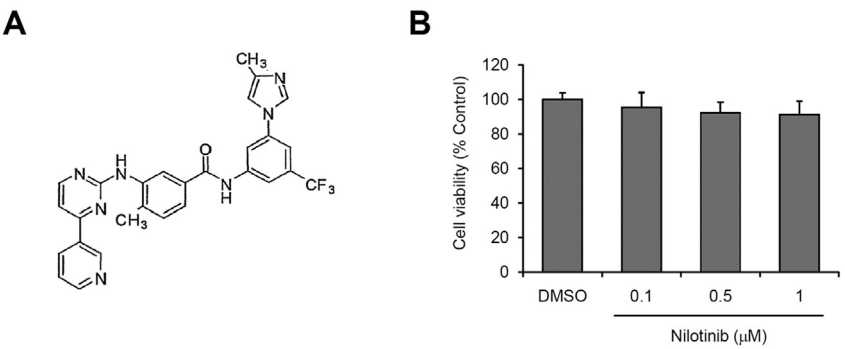

Fig. 1. (A) Structure of nilotinib. (B) Cytotoxicity of nilotinib in HM3KO melanoma cells. Cells were treated with the indicated concentrations of nilotinib for 24 h. Cytotoxicity was measured by MTT assay. There was no cytotoxicity of nilotinib at the indicated concentrations. The data are represented as percent control (DMSO-treated group). The mean values ± SD are averages of triplicate measurements.

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