

Original Research Article

7-Piperazinethylchrysin inhibits melanoma cell proliferation by targeting Mek 1/2 kinase activity

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Abstract

Purpose: To investigate the growth-inhibitory effect of 7-piperazinethylchrysin (PEC) on melanoma cell lines.

Methods: Cell viability was analyzed by trypan blue exclusion assays and the cell cycle by flow cytometry using ModFit LT software. Specifically, cells were stained with propidium iodide (0.5 mg/mL) supplemented with RNase A (50 mg/mL), and analyzed using flow cytometry and ModFit LT software.

Results: In A375 and B16F10 cell cultures, proliferation was reduced to 79 and 72 %, respectively, on treatment with 30 μ M PEC. PEC increased the proportion of A375 cells in G1/G0 phase to 71.23 %, versus 42.76 % in untreated cells. In B16F10 and A375 cells, treatment with PEC caused the inhibition of Mek 1/2 kinase activity and suppressed Erk 1/2 phosphorylation. The level of cAMP-response element binding protein was increased by PEC. The expression of microphthalmia-linked transcription factor was also increased by PEC treatment. Marked enhancement was observed in the level of tyrosinase in melanoma cells on treatment with PEC. Analysis of PBG-D expression showed a marked increase in B16F10 and A375 cells on the addition of PEC to cell cultures at 72 h. The level of PBG D expression was increased by 9- and 8.5-fold in B16F10 and A375 cells, respectively, on incubation with 30 μ M PEC. The addition of a Mek 1/2 inhibitor (U0126) to the cultures promoted PEC-mediated growth inhibition.

Conclusion: PEC inhibited melanoma cell proliferation, apparently by blocking the cell cycle at G0/G1 and downregulating the Ras/Raf/Mek/Erk pathway.

Keywords: Tyrosinase, Kinase, Microphthalmia, Phosphorylation, 7-Piperazinethylchrysin

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INTRODUCTION

Human melanoma is one of the most commonly detected malignant cancers of the skin, and its incidence is increasing worldwide [1]. Melanoma is detected mainly in females in the age group of 25 – 29 years [2]. The average survival period for patients suffering from the metastatic stage of melanoma is only 7 months [3,4]. Currently, the primary treatment for melanoma consists of

surgery [3,4]. The use of chemotherapeutic agents is limited because of the development of drug resistance [5].

Many skin problems, including the formation of wrinkles and appearance of dark spots, can be treated successfully by the application of natural products [6,7]. Thus, natural products may also offer novel therapeutic agents for the effective treatment of melanoma.

Flavonoids are an abundant class of natural products in plants, and biological investigations have revealed their promising potential as anti-cancer agents [8]. Studies have demonstrated that flavonoids exhibit inhibitory effects, both *in vitro* and *in vivo*, on tumor cell proliferation in various types of cancers, including prostate, breast, and hepatic cancer cells [9].

An important advantage of flavonoids is their low toxicity in living systems. Thus, flavonoids are important candidates for the treatment of cancer.

5, 7-Dihydroxyflavone, commonly known as chrysin, is a flavonoid isolated from extracts of many plants, including *Passiflora caerulea*, and propolis [10]. The biological properties of chrysin include anti-inflammatory, anti-oxidant, and anti-cancer activities [11]. In cervical cancer and leukemia cells, chrysin treatment causes the induction of apoptosis [12]. *In vivo* studies have shown that chrysin prevents carcinoma metastasis by inhibiting tumor angiogenesis [13].

A derivative of chrysin, 7-piperazinethylchrysin (PEC), is a potent anti-cancer agent against various carcinoma cell lines [14]. The current study was designed to investigate the effects of PEC on melanoma cells and its mechanism of action.

EXPERIMENTAL

Reagents and test compound

PEC was synthesized according to Edward *et al* [15]. The compound was dissolved in methanol to prepare a stock solution, which was diluted to provide solutions of the desired concentration. A specific inhibitor of Mek 1/2, U0126, was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell lines and culture conditions

The murine B16F1 melanoma cell line was purchased from the National Center for Cell Sciences; the A375 human melanoma cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). Both lines were cultured in RPMI-1640 medium supplemented with 10 % fetal bovine serum. Cell culture was performed at 37 °C in a humidified atmosphere consisting of 5 % CO₂ and 95 % air.

Evaluation of cell viability

A375 and B16F10 cells were grown in 24-well plates at a density of 2×10^5 cells per well and cultured for 24 h. Following culture, various

concentrations of PEC, solvent, a combination of PEC and U0126, or U0126 were added to the wells, and the plates were incubated for 72 h. After incubation, the cells were washed, harvested, and subsequently subjected to trypsinization. Trypan blue solution (0.4 %) was then added to the wells followed by an examination of the cells under an optical microscope. The effects of PEC and U0126 on cell viability were compared with control cells treated with solvent alone.

Cell cycle analysis

The cells were distributed in 6-well plates at 3×10^4 cells per well and cultured for 24 h. After incubation, the cells were treated for 72 h with various concentrations of PEC or solvent alone (control). The cells were then collected, washed with PBS, and subsequently fixed in 70 % ethyl alcohol. The cells were then stained with propidium iodide (PI; 0.5 mg/mL), supplemented with RNase A (50 mg/mL), and analyzed using flow cytometry and ModFit LT software (ver. 4.0; BD Biosciences, Franklin Lakes, NJ, USA).

Determination of melanin formation

A375 cells were seeded in 24-well plates at 2×10^5 cells per well. After culture for 24 h, the cells were incubated with PEC or solvent for 72 h. After washing, the cells were collected and subjected to trypsinization. The cells were then centrifuged (700 × g, 20 min) to collect the supernatant for the determination of extracellular melanin. The supernatant (1 mL), after treatment with HEPES buffer (0.4 M, 6.8 pH) and 90 % ethyl alcohol, was subjected to optical density measurements at 476 nm. For the determination of intracellular melanin, treated cells were washed, trypsinized, and subsequently treated with 1 N NaOH. Melanogenesis was determined by the sum of the extracellular and intracellular melanin contents compared with control cells.

Evaluation of tyrosinase activity

After culture and incubation with PEC or solvent for 72 h, cells were collected, washed twice with PBS, and subjected to trypsinization. The cells were then treated with trypan blue (0.4 %). Cell lysates were prepared by treating the cells for 30 min with a solution of Triton X-100 (1 %) in sodium phosphate buffer (0.1 M, 6.8 pH). Incubation of the lysates was performed for 4 h with DOPA (3, 4-dihydroxyphenylalanine) and sodium phosphate buffer. After incubation, the absorbance was recorded at 490 nm to measure the activity of tyrosinase as the fold increase in comparison with control cells.

Western blot analysis

After treatment with PEC or solvent for 72 h, the cell lines were subjected to protein extraction on ice using lysis buffer (100 mM NaCl, 20 mM Tris HCl, pH 7.8, and 0.1 % NP-40). The cellular homogenates were subjected to centrifugation (10,000 rpm, 15 min, 4 °C) to obtain the supernatant, which was stored at -80°C until analysis. The total protein samples obtained were separated by 10 % SDS-PAGE and then transferred electrophoretically to nitrocellulose membranes. Blockage of non-specific sites on the membranes was performed at room temperature for 1 h using 2 % bovine serum albumin. Each membrane was incubated with primary antibodies overnight at 4 °C. The primary antibodies used were anti-p-Erk 1/2 (Tyr-204), anti-p-Mek 1/2 (Ser 218/Ser 222), anti-microphthalmia related transcription factor (Mitf) (N-15), anti-p-cAMP-response element binding protein (CREB)-1 (Ser 133), and anti-PBG-D (A-16) (all from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Following incubation, the membranes were washed and then incubated for 1 h with horseradish peroxidase-conjugated secondary antibodies at room temperature. After washing, the blots were developed using an enhanced chemiluminescence kit (Amersham, Buckinghamshire, UK) and subjected to autoradiographic film exposure.

Statistical analysis

All data are presented as mean \pm standard deviations. SigmaStat software (SPSS-Jandel 2.0 Scientific, San Jose, CA, USA) was used for all statistical analyses. An analysis of the raw data was performed using the unpaired Student's *t*-test. Among various doses, differences were considered statistically significant at $p \leq 0.05$.

RESULTS

PEC inhibited A375 and B16F10 cell proliferation

In A375 cell cultures, the addition of PEC reduced proliferation by 12, 27, 36, 45, 63, and 79 % at 5, 10, 15, 20, 25 and 30 μ M, respectively (Figure 1). The proliferation of B16F10 cells was also reduced by PEC in a concentration-dependent manner. B16F10 cell proliferation was reduced by 9, 18, 29, 38, 59, and 72 % on treatment with 5, 10, 15, 20, 25 and 30 μ M PEC, respectively, after 72 h.

PEC induced cell cycle arrest in A375 and B16F10 cells

Flow cytometry revealed that PEC increased the proportion of A375 cells in G1/G0 phase, with a consequent reduction in S phase (Figure 2). The proportion of A375 cells treated with 30 μ M PEC in G1/G0 phase was increased to 71.23 % in comparison with 47.76 % in untreated cells. In S phase, the A375 cell proportion decreased, to 11.09 %, on treatment with 30 μ M PEC, relative to 29.67 % in untreated cells. In G2/M phase, the percentage of A375 cells was 15.89 and 21.46 % in cultures treated with 30 μ M PEC and without treatment, respectively (Figure 2). Similar results were obtained with B16F10 cells (Figure 2).

PEC altered Mek 1/2 activity in melanoma cells

In A375 and B16F10 melanoma cell cultures, the addition of PEC caused a reduction in Erk phosphorylation after 72 h (Figure 3). The addition of 5, 10, 15, 20, 25 and 30 μ M PEC to cultures of A375 cells reduced the Erk phosphorylation level in a concentration-dependent manner.

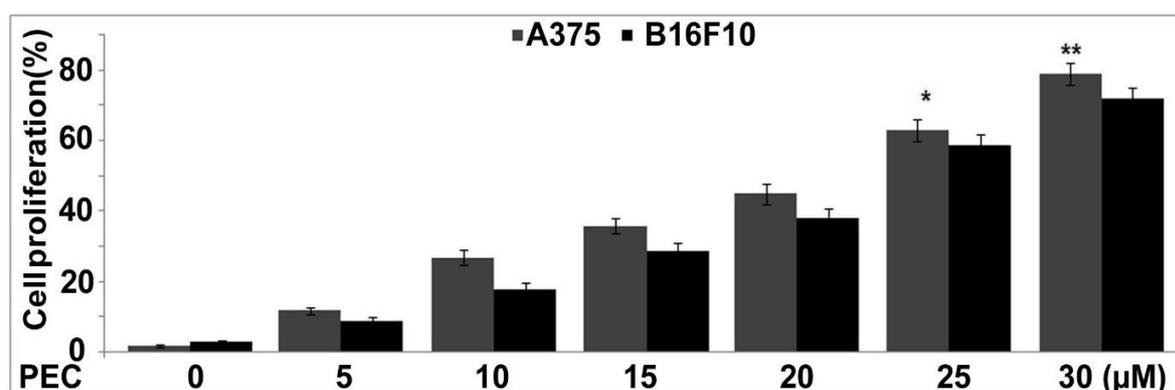


Figure 1: Effect of PEC on the rate of melanoma cell proliferation. Cells were treated with the indicated doses of PEC for 72 h followed by the determination of viability using trypan blue exclusion. The data are the means of triplicate experiments carried out independently; * $p < 0.005$ vs. untreated cell cultures

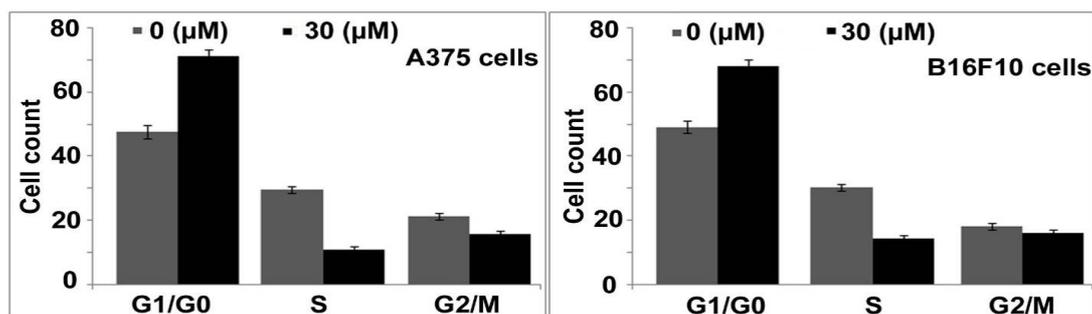


Figure 2: Effect of PEC on the cell cycle in melanoma cells. A375 and B16F10 cells were treated with PEC and then stained with PI. The data are from triplicate experiments. $**p < 0.001$ vs. untreated cells

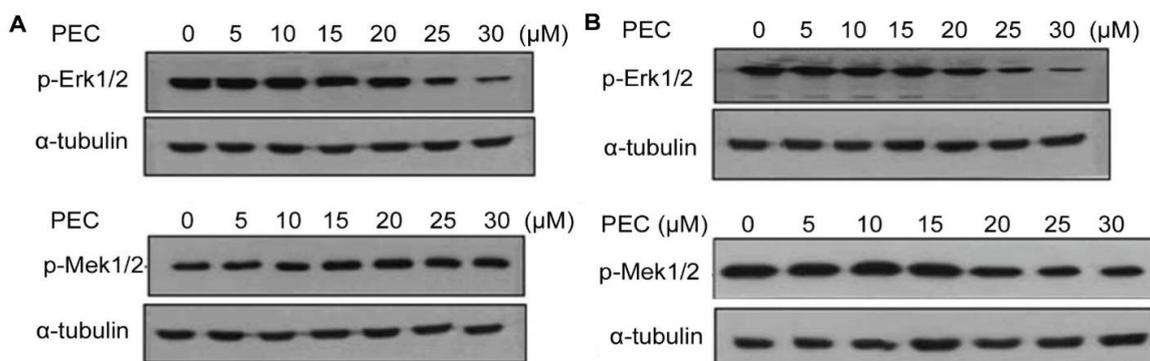


Figure 3: Effect of PEC on the phosphorylation of Erk 1/2 and activation of Mek 1/2 in melanoma cells. A Western blot assay was used to analyze the phosphorylation of Erk 1/2 and activation of Mek 1/2 in (A) A375 and (B) B16F10 cells after PEC treatment

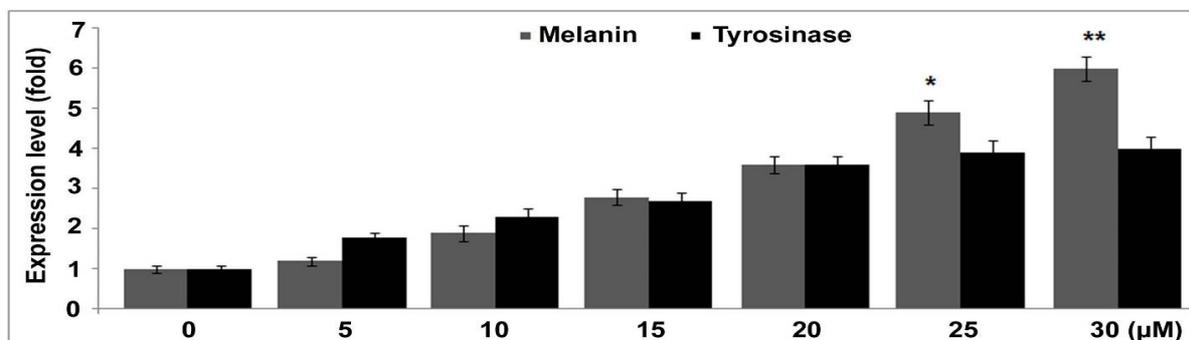


Figure 4: PEC promotes the formation of melanin and tyrosinase in A375 cells. The data are the means of three independent experiments; $*p < 0.005$, $**p < 0.005$ vs. solvent-treated cells

Similarly, in B16F10 cells, the phosphorylation level was reduced on treatment with 5, 10, 15, 20, 25 and 30 μM PEC. Mek 1/2 activation was promoted in both A375 and B16F10 cell cultures on the addition of PEC. Densitometry revealed 8, 19, 47, 69, 72 and 91 % increases in the activation level of Mek 1/2 in A375 cell cultures on the addition of 5, 10, 15, 20, 25 and 30 μM PEC, respectively.

Similarly, in B16F10 cell cultures, the addition of 5, 10, 15, 20, 25 and 30 μM PEC enhanced the Mek 1/2 activation level to 6, 16, 43, 61, 74 and 88 %, respectively (Figure 3A and Figure 3B).

PEC promoted melanogenesis in melanoma cells

In A375 cell cultures, incubation with PEC promoted the formation of melanin at 72 h (Figure 4). Melanin formation was increased by 1.2, 1.9, 2.8, 3.6, 4.9 and 6-fold in A375 cells upon treatment with PEC at 5, 10, 15, 20, 25 and 30 μM , respectively. Tyrosinase activity in A375 cells was also promoted, by 1.8, 2.3, 2.7, 3.6, 3.9 and 4-fold, on treatment with 5, 10, 15, 20, 25 and 30 μM PEC, respectively (Figure 4).

PEC increased the phosphorylation of CREB and the expression of Mitf in melanoma cells

In A375 cell cultures, the addition of PEC increased the CREB phosphorylation level at 72 h. PEC addition at 5, 10, 15, 20, 25 and 30 μM increased the phosphorylation of CREB in a dose-based manner versus untreated cells (Figure 5A). The level of Mitf was also increased in A375 cells on treatment with PEC for 72 h. The increase was maximal at 30 μM PEC. Similar results were observed for CREB and Mitf in B16F10 cells (Figure 5B).

PEC increased the level of porphobilinogen deaminase (PBG-D) in melanoma cells

An analysis of the expression level of PBG-D protein showed a marked increase in A375 and B16F10 cells on the addition of PEC to cell cultures at 72 h. The level of PBG-D expression was increased, 2.0, 3.5, 4.6, 5.9, 7.6 and 9-fold, by 5, 10, 15, 20, 25 and 30 μM PEC in A375

cells, respectively (Figure 6A). Similarly, in B16F10 cells, the addition of PEC to cultures increased the level of PBG-D at 5, 10, 15, 20, 25 and 30 μM by 1.9, 3.1, 4.4, 5.3, 7.4 and 8.5-fold, respectively (Figure 6B).

U0126 promoted the PEC-mediated inhibition of A375 and B16F10 cell proliferation

In A375 cell cultures, the addition of PEC reduced cell growth by 79 % at 30 μM (Figure 7). Addition of the Mek 1/2 inhibitor U0126 enhanced the PEC-mediated reduction in cell growth. PEC at 30 μM was added to cultures of A375 cells in combination with various concentrations of U0126. At 1, 2, 4, 6, 8 and 10 μM U0126, in combination with PEC, cell viability was reduced by 83, 87, 89, 92, 93 and 96 %, respectively, versus the control (Figure 7). Similar results were observed for the inhibition of B16F10 cell proliferation by U0126 treatment (Figure 7).

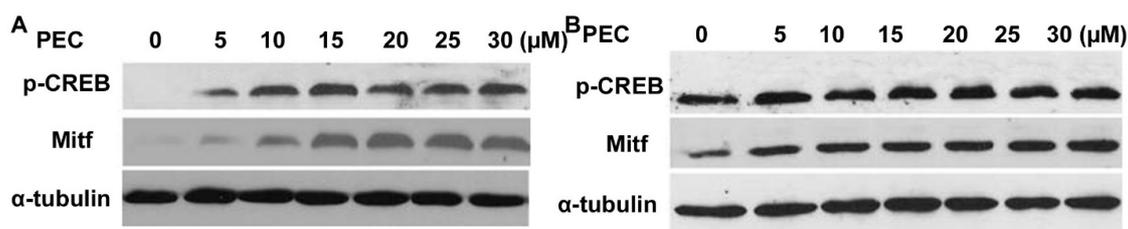


Figure 5: Effect of PEC on the phosphorylation of CREB and expression of Mitf in melanoma cells. (A) A375 and (B) B16F10 cells were treated with PEC for 72 h and then analyzed for changes in CREB phosphorylation and Mitf expression using Western blotting

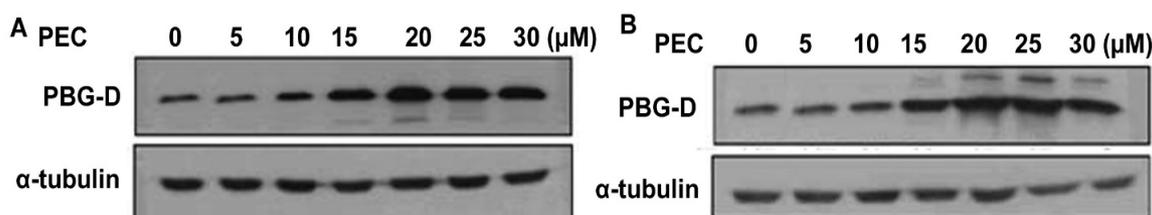


Figure 6: Effects of PEC on the PBG-D levels in melanoma cells. Cells treated with PEC were analyzed using Western blotting to assess the levels of PBG-D. α -Tubulin was used as an internal loading control

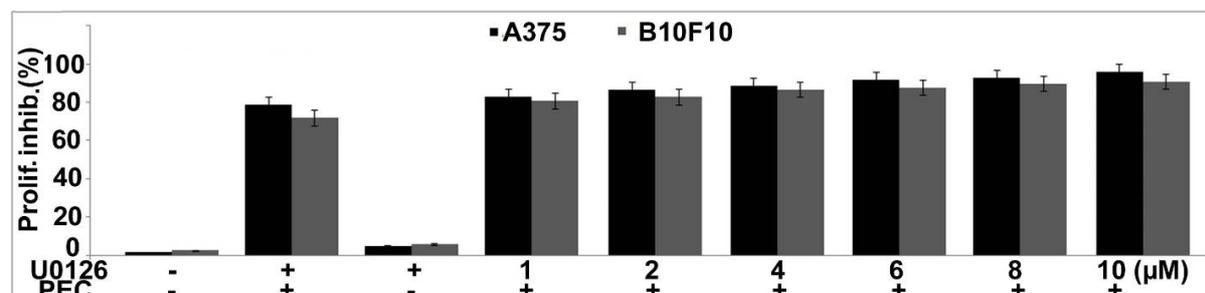


Figure 7: Effect of the Mek 1/2 inhibitor U0126 on the PEC-mediated reduction in cell growth. Cells were treated with the indicated doses of U0126 and PEC followed by an analysis of cell viability. The data are the means of triplicate independent experiments; * $p < 0.005$ vs. untreated cell cultures

DISCUSSION

In this study, we demonstrated the role of PEC in the inhibition of melanoma cell growth. The data show that PEC inhibited melanoma cell proliferation and blocked the cell cycle in G0/G1.

The regulation of cellular processes, including viability, proliferation, and differentiation, via the transfer of signals from the cell to the nucleus, leading to cell cycle progression at G0/G1, is maintained by Erk 1/2, which belongs to the mitogen-activated protein kinase (MAPK) signaling pathway [16]. MAPK pathway regulation and Erk activation are dependent on Mek 1/2. The present study indicates that Erk phosphorylation was inhibited in melanoma cell lines upon treatment with PEC. PEC treatment also promoted the activation of Mek 1/2 in melanoma cells. Studies have shown that, among melanoma cells, more than 60 % harbor an activating mutation that induces Mek and Erk activation and consequently promotes the proliferation of cells and inhibits pigmentation [17].

The current study demonstrates that PEC treatment promoted melanogenesis in melanoma cells in a dose-dependent manner. Thus, PEC promoted Mek 1/2 activation, reducing its kinase activity and leading to the upregulation of melanogenesis through a cell cycle arrest. The formation of melanin in melanoma cells is induced via cAMP [18]. The activation and translocation of protein kinase A, and, consequently, CREB activation and gene transcription, are due to cAMP [19]. One of the genes expressed during this process is Mitf, which plays important roles in the regulation of melanocyte viability, proliferation, and differentiation [20].

Mitf is also involved in the transcription of tyrosinase-related genes, which are involved in melanogenesis induced by cAMP [21]. In the current study, PEC treatment significantly promoted the formation of melanin and the activity of tyrosinase in melanocytes. In melanoma cell lines, the addition of PEC increased the phosphorylation level of CREB. The level of Mitf was also increased in melanoma cells on treatment with PEC. Studies have shown that Erk 1/2 activation leads to the downregulation of Mitf expression [22]. Thus, the present study shows that PEC promoted the expression of Mitf and increased the CREB level by downregulating Erk 1/2 phosphorylation. In melanoma cells, Mitf mediated melanogenesis via an arrest of the cell cycle in G1/G0 phase.

In the present study, PEC treatment inhibited the activity of Mek 1/2 kinase and phosphorylation of Erk, which play key roles in cell proliferation-related gene transcription. Thus, it appears that PEC treatment causes stabilization of the cAMP signaling pathway in melanoma cells, phosphorylates CREB, and increases the level of Mitf, leading to the promotion of differentiation and melanogenesis. Our results show that U0126, in combination with PEC, enhanced the inhibition of melanoma cell proliferation. U0126 inhibited cell viability, promoted melanoma cell differentiation, and inhibited tumor cell invasion [23]. Major factors that mediate the Raf signaling pathway include Mek 1 and Mek 2, which are considered to be of great value for downregulating the Raf/Mek/Erk pathway in carcinomas [23]. This study thus shows that U0126 downregulated the Ras/Raf/Mek/Erk pathway, which, in turn, increased the sensitivity of melanoma cells to PEC.

CONCLUSION

PEC inhibited the proliferation of melanoma cells by blocking the cell cycle at G1/G0 phase and induced melanogenesis through Mek 1/2 activation. Thus, PEC may be useful for the induction of differentiation and melanogenesis in melanoma cells.

DECLARATIONS

Acknowledgement

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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