A novel psoralen derivative-MPFC enhances melanogenesis via activation of p38 MAPK and PKA signaling pathways in B16 cells

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Abstract. As an active compound, psoralen is present in various Chinese herbal medicines and has exhibited significant activity in skin disease treatment. Its derivative 8-methoxypsoralan (8-MOP) is the most commonly used drug to induce repigmentation of vitiligo. In our previous screening assays, 4-methyl-6-phenyl-2H-furo[3,2-g]chromen-2-one (MPFC), a psoralen derivative, was identified as more effective tyrosinase and melanin activator than the positive control 8-MOP in consideration of low doses, as well as low toxicity. The overall purpose of this study was to characterize the melanogenic effect and mechanisms of MPFC in B16 cells. The melanin biosynthesis effects of MPFC were determined by examination of cellular melanin contents, tyrosinase activity assay, cyclic adenosinemonophosphate (cAMP) assay, and western blotting of MPFC-stimulated B16 mouse melanoma cells. Our results showed that MPFC enhanced both melanin synthesis and tyrosinase activity in a concentration-dependent manner as well as significantly activated the expression of melanogenic proteins such as tyrosinase, tyrosinase-related protein-1 and tyrosinase-related protein-2. Western blot analysis showed that MPFC increased the phosphorylation of p38 mitogen-activated protein kinase and cAMP response element-binding protein (CREB) as well as the expression of microphthalmia-associated transcription factor (MITF). Moreover, MPFC stimulated intracellular cAMP levels and induced tyrosinase activity and melanin synthesis were attenuated by H89, a protein kinase A inhibitor. These results indicated that MPFC-mediated activation of the p38 MAPK and the protein kinase A (PKA) pathway may shed light on a novel approach for an effective therapy for vitiligo.

Introduction

Leukoderma, also named vitiligo, is an acquired disfiguring pigimentary anomaly of the skin manifested by depigmented white patches surrounded by a normal or hyper-pigmented border (1). Vitiligo affects 1-2% of the world population without racial or sex difference (2). Hypopigmentation may be the source of severe psychological distress, diminished quality of life, and increased risk of psychiatric morbidity (3). However, the effective medicines are largely absent from the clinical treatment of the disease.

Melanin synthesis in the skin plays an important evolutionary role in hypopigmentation therapy. In mammals, melanin biosynthesis is catalyzed by three melanocyte-specific enzymes: tyrosinase (TYR), tyrosinase-related protein 1 (TRP1), and TRP2 (4). TYR is the rate-limiting enzyme in melanogenesis (5), catalyzing the hydroxylation of tyrosine to produce 3,4-dihydroxyphenylalanine (DOPA) and the oxidation of DOPA to DOPA quinone. TRP-1 and TRP-2 function in the biosynthesis of melanin downstream of TYR (4). Microphthalmia-associated transcription factor (MITF) has a crucial role in the transcription of melanogenic genes, binding a highly conserved motif termed M-box within the TYR promoter. Thereby MITF plays an important role in increasing melanogenesis (6,7).

Many approaches have been used to help clarify the specific mechanism controlling melanin biosynthesis via tyrosinase regulation. The mitogen-activated protein kinases (MAPKs) are key signaling molecules related to the regulation of melanogenesis (8), including extracellular signal-regulating kinase (ERK), stress-activated protein kinase (SAPK)/JNK and p38 mitogen-activated protein kinase (p38 MAPK) signaling cascades. Previous literature showed that phosphorylation of p38 can lead to the activation of MITF via the phosphorylation of cyclic adenosinemonophosphate (cAMP) responsive element binding (CREB) protein. Some Chinese medicine extracts such as methyl 3,5-di-caffeoylquinlate have been shown to have melanogenesis activity through activating the p38 signaling pathway (9).
Another signaling pathway involved in melanogenesis regulation is protein kinase A (PKA). PKA can be activated by the elevation of cellular cAMP and cAMP stimulation results in the elevation of MITF protein levels and subsequent activation of the TYR, TRP-1 and TRP-2 promoters by binding with M-box or E-box consensus motif (10,11).

Previous literature also showed that the inhibition of the PI3K/AKT pathway increases the production of melanin by MITF activation and induction of tyrosinase expression (12).

As an active compound, psoralen is present in a variety of traditional Chinese medicinal plants, such as *Psoralea corylifolia* L., *Glehnia littoralis* Fr. Schmid ex Miq, *Heracleum lanatum* Michx., *Ruta graveolens* L. and *Ficus carica* L. Recent studies have revealed that it possesses significant pharmacological activities in dermatosis treatment, including vitiligo, psoriasis and alopecia areata. Similarly, the extract of *Psoralea corylifolia* L. seeds was one of the most popular Uygar medicines used for vitiligo and initially recorded in ‘Yao Yong Zong Ku’ around 300 years ago (13-15). In 1930s, 8-methoxypsoralen (8-MOP) and 5-methoxypsoralen (5-MOP) were isolated from the *Psoralea corylifolia* L. (16,17). Later, other psoralens, such as 4,5,8-trimethylpsoralen (TMP) were synthesized as well. Continuous research proved that these compounds show strong activity in the treatment of vitiligo (13,14). Among them, 8-MOP is considered as a better therapeutic agent against vitiligo in consideration of low doses and toxicity. However, it is still accompanied by some undesired side effects in clinical therapy, such as gene mutation, skin phototoxicity and risk of skin cancer (18,19). So, it is necessary to find substitutions for enhancing skin hypopigmentation.

Our group has been dedicated to the drug development of vitiligo for many years (20-23). Recently, a new series of furocoumarin derivatives were designed and synthesized by our research team (20), and biologically evaluated for activity on tyrosinase and melanin synthesis in murine B16 cells. 4-Methyl-6-phenyl-2H-furo[3,2-g]chromen-2-one (MPFC) (Fig. 1) is regarded as one of the most promising candidate compound with an effect on melanin synthesis and tyrosinase activity much better than the positive control 8-MOP. We speculate that better melanogenesis activity of MPFC may result from the different structural modifications compared with 8-MOP. In this study, we evaluated the activity of MPFC on melanogenesis and provide solid evidence showing that p38 MAPK and PKA pathway are targets of this compound to active melanin biosynthesis.

**Materials and methods**

**Reagents.** Dimethylsulfoxide (DMSO) was from Sigma (St. Louis, MO, USA), 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium (CCK-8) was purchased from TransGen Biotechnology (Beijing, China). CERB, phospho-CREB (Ser133), AKT, p-AKT (Thr308), p38, p-p38 (Thr180/Tyr182), ERK, p-ERK (Thr202/Tyr204), JNK, p-JNK (Thr183/Tyr185) and β-actin antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies against tyrosinase, TRP1 and TRP2 were from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Anti-MITF antibody was purchased from Millipore (Billerica, MA, USA). Anti-mouse, anti-goat and anti-rabbit IgG antibodies (horseradish peroxidase conjugated) were purchased from Santa Cruz Biotechnology, Inc.

**Preperation of MPFC (20).** Four percent ethanol potassium hydroxide solution (70 ml) was added to an ethanolic solution (500 ml) of intermediate 4-methyl-7-(2-oxo-2-phenylethoxy)-2H-chromen-2-one (10 mmol), and the mixture was refluxed for 4 h. After cooling, the solution was acidified with 1 M hydrochloric acid and extracted with ethyl acetate three times. The organic phase was dried overnight and evaporated under reduced pressure. The resulting residue was purified by silica gel chromatography with petroleum ether/ethyl acetate to obtain MPFC. Yield 97%, light yellow solid, m.p. 171-173°C; purity 98.70%; ¹H NMR (400 MHz, CDCl₃) δ 7.98 (s, 1H), 7.83 (s, 1H), 7.64 (dd, J = 8.2, 1.1 Hz, 2H), 7.50-7.56 (m, 3H), 7.44 (td, J = 7.4, 1.1 Hz, 1H), 6.29 (s, 1H), 2.52 (s, 3H). ¹³C NMR (101 MHz, CDCl₃) δ 160.99 (s), 157.10 (s), 152.64 (s), 151.80 (s), 142.88 (s), 131.06 (s), 129.25 (s), 128.06 (s), 127.58 (s), 123.96 (s), 122.32 (s), 116.80 (s), 115.77 (s), 113.64 (s), 100.23 (s), 19.26 (s). IR (KBr) v: 2925, 1735, 1611, 1447, 1280, 1125, 1063, 831 cm⁻¹; HRMS (ESI) calcd for C12H12O3 [M+H]+ 277.0853, found 277.0853. MPFC was dissolved in DMSO and stored at -20°C as a stock solution (50 mM).

**Cell culture.** The murine B16 melanoma cell line (acquired from Chinese Academy of Sciences, Beijing, China) were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS), penicillin G (100 U/ml) and streptomycin (100 mg/ml) (Gibco-BRL, Grand Island, NY, USA) at 37°C in a humidified atmosphere of 5% CO₂.

**Cell morphology and cell viability measurement.** Cell morphology was examined under a LEICA DMi8 microscope (LEICA Microsystems CMS GmbH, Wetzlar, Germany). Cell viability was assayed by adding CCK-8 (TransGen Biotech) solution. Briefly, B16 cells were plated in 96-well dishes at a density of 5x10⁶ cells/well and allowed to adhere for 24 h. Test samples were added and the cells were incubated for 24 h. After discarding the culture medium of the cells, 10 µl of CCK-8 solution was added into each well and cells were incubated at 37°C for another 2 h. The absorbance was determined at 450 nm using a Spectra Max M5 (Molecular Devices, Sunnyvale, CA, USA). Absorbance of cells without treatment was regarded as 100% of cell survival. Each treatment was performed in triplicate and each experiment was repeated three times.

**Tyrosinase activity assay.** Tyrosinase activity was estimated by measuring the rate of L-DOPA oxidation. Briefly, B16 cells were seeded in a 6-well plate at a density of 2x10⁵ cells/well and allowed to attach for 24 h. Test samples were then added to individual wells. After a 24 h incubation, cells were washed with ice-cold phosphate-buffered saline (PBS) twice, lysed with 1% Triton X-100 solution containing 1% sodium deoxycholate for 30 min at 80°C, then the lysate was centrifuged at 12,000 x g for 15 min to obtain the supernatant. A reaction mixture containing 10 mM L-DOPA in PBS (pH 6.8) was added and then, the cells were incubated at 37°C in dark for...
Melanin measurement. B16 cells were seeded in a 6-well plate at a density of 2x10^5 cells/well and allowed to attach for 24 h. Then adding test samples to individual wells, cells were incubated for 48 h and washed with PBS. After cells were lysed according to the method previously described (9), lysate was put in a 96-well microplate, and measured spectro-photometrically at 405 nm by a multi-plate reader. Protein concentration of each sample was determined by BCA Protein assay kit (Biomed, Beijing, China). The melanin amount expressed as abs/µg protein was shown as percentage value. The percentage value of the treated cells was calculated with respect to the untreated cells. Each experiment was repeated three times.

Measurement of cAMP concentration. The cAMP level was measured using a cAMP ELISA kit (Cell Biolabs, Inc., San Diego, CA, USA) B16 cells were plated in a 6-well plate at a density of 5x10^5 cells/well and allowed to adhere for 24 h, then test samples were added to individual wells. After incubating for 12 h, B16 cells were harvested and lysed in lysis buffer. Supernatants were collected after centrifuging to determine cAMP levels using a commercially available cAMP ELISA kit. cAMP levels were normalized to total protein content. Each experiment was repeated three times.

Western blot analysis. The treated cells were lysed in cold RIPA buffer (pH 7.4) containing protease and proteinase inhibitor cocktail (1 M 4-nitrophenyl phosphate disodium salt hydrate (PNPP), 1 M sodium fluoride (NaF), 10 mM phenylmethanesulfonyl fluoride (PMSF), 100 mM benzamidine, 100 mM DL-Dithiothreitol (DTT), 200 mM sodium orthovanadate (OV)). The whole-cell lysate was collected and regarded as a protein sample. Its concentration was measured by BCA Protein assay kit. The targeted proteins were detected by ECL western blot antibodies at a dilution of 1:2,000 for 1 h at room temperature. Following electrophoresis in a polyvinylidene fluoride (PVDF) membranes (Merck Millipore Ltd., Darmstadt, Germany) membrane blocking was performed with 5% skim milk solution for 1 h, then they were incubated with horseradish peroxidase-conjugated secondary antibodies at a dilution of 1:2,000 for 1 h at room temperature. The targeted proteins were detected by ECL western blot detection reagents (GE Healthcare, Pittsburgh, PA, USA), and visualized after exposure to chemiluminescence film (X-OMAT BT film; Carestream Health, Inc., Xiamen, China). Western blot assay results reported here are representative of at least three experiments.

Statistical analysis. Data were expressed as the mean ± SD and statistical analysis was performed with one-way ANOVA followed by Tukey post hoc test for multiple comparison tests. A P-value of <0.05 was considered of significant difference.

Results

Morphological changes of melanoma cells induced by MPFC. Our results showed that murine melanoma B16 cells treated with MPFC for 24 h did not induce any change in cell morphology when compared with the untreated cells (Fig. 2A) and did not show any increase in cytotoxicity (Fig. 2B). Thus, dosages at 0-50 µM were chosen to determine the effects of MPFC on tyrosinase activity and melanin synthesis.

Treatment with MPFC stimulates tyrosinase activity and melanin content in B16 cells at non-cytotoxic dosages. Treatment with MPFC demonstrated the increased tyrosinase activity in a dose-dependent manner. At the same concentration of 50 µM, the tyrosinase activity of MPFC was increased by 20% compared with 8-MOP (0 µM, 100±14.4%; 12.5 µM, 108.1±3.9%; 25 µM, 125.9±10.6%; 50 µM, 149.8±3.9%; 8-MOP, 50 µM, 129.6±6.9%) (Fig. 3A). As shown in Fig. 3B, melanin amount showed the same increasing trend in response to MPFC treatment, and the melanin content of MPFC was increased 90% more than 8-MOP at 50 µM (0 µM, 100±14.4%; 12.5 µM, 100±14.4%; 25 µM, 109.7±3.5%; 25 µM, 134.6±3.6%; 50 µM, 213.3±16.4%; 8-MOP, 50 µM, 124.2±2.4%). These results provide a pharmacological basis for the traditional use of MPFC instead of 8-MOP in melanogenesis.

Effect of MPFC on the expression of TRPs. Since MPFC increased tyrosinase activity and melanin production, we explored the melanogenic signaling pathway related to the stimulatory activity of MPFC. After treatment with MPFC, the expression of melanogenesis-related proteins (MRPs) such as tyrosinase, TRP1, and TRP2 was examined by western blotting. MRP expression was clearly increased after treatment with MPFC in a dose-dependent manner (Fig. 4).

MPFC induces CREB activation and enhances the expression of p-MITF. In order to elucidate how MPFC activates melanin synthesis, both CREB and MITF were hypothesized to be involved in MPFC-induced melanogenesis. As expected,
the expression of phosphorylated MITF by MPFC treatment for 48 h had a significant increase. Our results also showed that phosphorylation of CREB was clearly enhanced for 1 h (Fig. 5) compared with cells treated with 0.1% DMSO only, which suggested that MPFC-mediated elevation of the MITF may be cAMP-dependent.

**MPFC induces melanin synthesis through intracellular cAMP accumulation and melanogenesis-related signaling pathways PKA.** To evaluate the hypothesis above, we explored whether MPFC affects the accumulation of cAMP, which is a vital step in melanogenesis. As seen in Fig. 6A, 12 h after MPFC addition, the level of cAMP was increased. cAMP-related biological effects depend on PKA, which has a direct influence on melanogenesis. Thus, we evaluated the effect of H-89 (Beyotime Biotechnology, Shanghai, China), an inhibitor of cAMP-dependent PKA, on the MPFC-mediated induction of tyrosinase activity and melanin content. As shown in Fig. 6B, MPFC-induced enhancement of tyrosinase activity on incubation for 24 h was abrogated by H-89. In addition, the melanin content after MPFC treatment for 48 h also reduced by H-89 compared with untreated cells (Fig. 6C). Generally, these results revealed the critical involvement of cAMP/PKA signaling in MPFC-mediated melanogenesis in B16 melanoma cells.

**MPFC induces activation of p38 MAPK.** The phosphorylation of MAPK or inhibition of PI3K/AKT activation was reported...
to be the signaling process in hyperpigmentation. Thus, we performed western blot analysis to determine the impact of MPFC on p38, ERK, JNK and AKT phosphorylation. As shown in Fig. 7, phosphorylation of p38 MAPK was significantly increased after 1 h at different concentrations of MPFC treatment compared with untreated cells. In contrast, no significant upregulation of AKT phosphorylation was induced by MPFC.

Effects of inhibitors of MAPKs and AKT on MPFC-induced tyrosinase activity and melanin content. Even co-treatment with ERK inhibitor (PD98059), JNK inhibitor (SP600125) (Beyotime Biotechnology) or AKT inhibitor IV (EMD Biosciences, Inc., Madison, WI, USA), MPFC-induced tyrosinase activity and melanin content were not influenced. However, the p38 MAPK inhibitor SB203580 (Beyotime Biotechnology) significantly reduced MPFC-triggered tyrosinase activity and melanin content (Fig. 8). These observations reveal that p38, but not ERK, JNK or AKT pathway, was directly involved in the upstream pathway of melanogenesis mediated by MPFC.

Discussion

In hypopigmentation therapy (24,25), the induction of melanin production was the focus of study to develop effective treatments (2,3,26,27). Natural resources have been screened and active compounds have been synthesized for the development of pigmentation agents by our research group, including chlorogenic acid (22), kaliziri extracts (23), furocoumarin derivatives (20) and isoxazole chalcone derivatives (21).

Recently, Niu et al. (20) synthesized a series of furocoumarin derivatives and discovered that many of them have strong activities in melanogenesis. In consideration of the generally low cytotoxicities of these compounds, we tested all of the derivatives in B16 melanoma cells, and identified that MPFC, a psoralen derivative, was an effective tyrosinase activator in our recent report. The melanin content and tyrosinase activity increased by 90 and 20%, respectively, in B16 cells treated with MPFC compared with 8-MOP treated controls in our research. We infer that the in vitro melanin synthesis evaluation of these structurally diverse analogues attributed to an outline of structure-activity relationship. Studies (31,32) have reported that 8-MOP leads to dramatic increases in melanin production through activating the protein kinase A and/or protein kinase C signaling pathways. By comparison, the regulation of MPFC in melanin synthesis results from cross-talk between several different signaling pathways.

As mentioned in the introduction, phosphorylation of MAPK (including ERK, JNK and p38 MAPKs) or inhibition of PI3K/AKT activation has been reported as one of the signaling processes in hyperpigmentation (33). It has been shown that p38 MAPK activates MITF through the phosphorylation of CREB, which in turn upregulates the expression of tyrosinase, TRP-1 and TRP-2, resulting in melanin production (34,35). Activations of the ERK signaling (36) and the JNK/SAPK pathways (37) are related to the downregulation of melanogenesis. Another signaling pathway involved in melanogenesis regulation includes phosphatidylinositol 3-kinase (PI3K)/AKT signaling, which phosphorylates MITF and promotes its activation, leading to melanogenesis enhancement (38,39). In our experiments, treatment with MPFC did not affect the total protein levels of ERK, JNK, AKT or p38. However, it significantly promoted the levels of p-p38, although not p-ERK, p-JNK and p-AKT in B16 cells. To verify whether p38 MAPK signaling factors are responsible for MPFC-induced activation effects on melanogenesis, co-incubation with p38 MAPK inhibitor SB203580 clearly abrogated MPFC-stimulated melanin content and tyrosinase activity. Unlike its effect on p38 MAPK, other inhibitors did not influence the MPFC-stimulated melanogenic process. These results suggested that p38 MAPK is responsible for the pigmentation process mediated by MPFC in melanoma cells among the upstream pathways involved in melanogenesis. The activation effects on melanogenesis of MPFC and phosphorylation of p38 demonstrated in our research are consistent with the above mentioned role of p38 signaling pathway in hyperpigmentation.

Figure 4. Representative western blot analyses illustrating expression of tyrosinase-related proteins (TRPs). B16 cells were treated with 4-methyl-6-phenyl-2H-furo[3,2-g]chromen-2-one (MPFC) at 0, 12.5, 25 and 50 µM for 48 h. Tyrosinase, TRP1 and TRP2 protein expression were detected by western blotting. Results were normalized against β-actin expression.

Figure 5. Induction of microphthalmia-associated transcription factor (MITF) upregulation and cAMP response element-binding protein (CREB) activation by 4-methyl-6-phenyl-2H-furo[3,2-g]chromen-2-one (MPFC). After incubation of B16 cells with MPFC at 0, 12.5, 25 and 50 µM for 24 h (MITF expression) or 15 min (CREB activation), western blotting was carried out using specific antibodies against phospho-MITF (p-MITF), total (MITF), phospho-CREB (p-CREB), total (CREB) and β-actin.
The PKA-dependent signaling pathway has also been reported as one of the signaling processes in hyperpigmentation (40). There is evidence that intracellular cAMP promotes MITF expression via phosphorylating the CREB family transcription factors. Once phosphorylated, CREB can upregulate MITF and subsequently results in the indirect activation of the tyrosinase promoter by MITF (41). In accordance with previous studies, we observed that MPFC induces the phosphorylation of CREB and enhances the production of cAMP compared with untreated cells. It is noteworthy that 8-MOP showed the same increasing trend in response to MPFC treatment in cAMP level, the results agreed with the experimental results from literature (31,32). H-89, an inhibitor of protein kinase A, completely abolished tyrosinase expression in B16 cells induced by MPFC, indicating that MPFC-mediated MITF activation relies on PKA signaling pathway.

In conclusion, MPFC enhanced melanin synthesis and tyrosinase activity through accelerating p38 MAPK and PKA signaling pathways. These results provide a molecular function for psoralen derivative components in melanogenesis and will help expand our knowledge of clinical therapy for enhancing skin hyperpigmentation.
Figure 8. Effects of inhibitors of MAPKs and AKT on 4-methyl-6-phenyl-2H-furo[3,2-g]chromen-2-one (MPFC)-induced tyrosinase activity and melanin content. Inhibitors (PD98059, SP600125 and SB203580 10 µM, AKT inhibitor IV 1 µM) were pre-incubated with B16 cells for 2 h before addition of MPFC at 30 µM, followed by an additional incubation for 24 or 48 h for tyrosinase activity and melanin content, respectively. Each percentage value in the treated cells was calculated with respect to that in the untreated cells. Values are expressed as the mean ± SD of three separate experiments. *P<0.05, **P<0.01 and ***P<0.001 compared with control; #P<0.05 and ##P<0.01 compared with MPFC stimulation.
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Availability of data and material

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LY, GP and CN conceived and designed the experiments and wrote the paper. LY and CN performed the experiments. GP, HAA and JD analyzed the data. HAA revised the paper. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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