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Chrysophanol induces cell apoptosis and suppresses cell invasion by regulating AKT and MAPK signaling pathway in melanoma cells

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ABSTRACT: Melanoma is a highly heterogeneous and invasive tumor with poor prognosis due to its resistance to radiotherapy, chemotherapy, and immunotherapy. Chrysophanol has the anti-carcinogenic role in a variety of tumors. Although the anti-proliferative role of chrysophanol in melanoma has been reported, its specific molecular mechanisms are still unclear. In the present study, the proliferation, migration, invasion, apoptosis, and cell cycle of A375 and A2058 cells were evaluated through MTT, colony formation, cell scratch, Transwell, and flow cytometry assays. Besides, the relative levels of proteins related to AKT and MAPK signaling pathway were determined by western blotting. The results revealed that chrysophanol significantly inhibited cell viability, colony formation, and cell invasive but increased the relative wound width of A375 and A2058 cells. Chrysophanol also notably enhanced the apoptotic rate, accompanied with the induced protein levels of Bax and Cleaved caspase-3 and reduced protein level of Bcl-2. In addition, chrysophanol observably promoted the ratio of G0/G1 phase, while restrained the relative protein expressions of Cyclin D1 and CDK4. Furthermore, the relative expressions of p-AKT/AKT, p-ERK1/2/ERK1/2, and p-JNK/JNK were significantly decreased, and the relative expression of p-38/38 was enhanced by 20, 50, and 100 μM chrysophanol treatment. Totally, the dose-dependent effects of chrysophanol on melanoma cell proliferation, migration, invasion, apoptosis, and cell cycle were observed. To sum up, these results revealed that chrysophanol induced cell apoptosis and suppressed cell invasion by regulating AKT and MAPK signaling pathway in both A375 and A2058 cells in a dose-dependent manner.

KEYWORDS: melanoma, chrysophanol, apoptosis, invasion, AKT, MAPK

INTRODUCTION

Cutaneous melanoma (CM) is one of highly invasive malignancies in the world, which differs from other types of melanomas (such as mucosal and ocular melanomas) in terms of risk and predictive and prognostic factors [1]. Although CM is the third leading skin malignancy, whose mortality rate is as high as 65% with a continued rise on the annual incidence and mortality [2], metastasis to lung, lymph nodes, and brain is an important determinant of the rise in melanoma-associated mortality [3]. Conventional melanoma therapies such as radiation therapy, surgery, chemotherapy, and targeted therapy as well as immunotherapies, including immune checkpoint blockade therapy, oncolytic viruses used to treat malignant melanoma, and the used of T-cell therapy have been demonstrated to be the significant and effective therapeutics for the treatment of melanoma [4]. Nevertheless, resistant to radiotherapy, chemotherapy, and immunotherapy results in a five-year survival rate less than 20% for patients with metastatic melanoma [5]. Thus, a more in-depth exploration of the pathogenesis of melanoma and the search for new therapeutic targets and drugs play a crucial role in prolonging the survival of melanoma patients.

Chrysophanol ($C_{15}H_{10}O_4$), also designated as 1,8-dihydroxy-3-methyl-anthraquinone, is one of the naturally occurring anthraquinone compounds that can be separated from a variety of traditional Chinese medicines such as *Cassia obtusifolia* L., *Rheum palmatum*., *Senna racemosa* (Mill.) H.S. Irwin & Barneby (Fabaceae), and *Radix et Rhizoma* Rhei [6]. Plenty of studies have elaborated the pharmacological effects of chrysophanol, including anti-inflammatory [7], anti-fibrotic [8], antibacterial [9], neuroprotective [10], and anti-diabetes [11] activities, which have generally shown effective roles in various diseases. Besides, the inhibitory effects of chrysophanol on diverse tumors have received more and more attention in recent years. For instance, chrysophanol was shown to inhibit the growth of breast cancer cell via the induction of endoplasmic reticulum (ER) stress and mitochondrial apoptosis [12]. Chrysophanol showed the anti-carcinogenic effect on human epithelial ovarian cancer cells via the activation of p38 mitogen-activated protein kinase (MAPK) and the overload of mitochondrial calcium [13]. The potentially suppressive role of chrysophanol was also observed in choriocarcinoma [14]. Though the anti-proliferative role of chrysophanol in melanoma A375 cell line was reported by Zhang et al [15], its specific molecular mechanisms are still unclear. Therefore, the anti-carcinogenic effects of chrysophanol and its associated

mechanisms in A375 and A2058 melanoma cell lines were investigated in the present study.

MATERIALS AND METHODS

Cell culture

A375 (CL-0014) and A2058 (CL-0652) melanoma cell lines were obtained from Procell (Wuhan, China). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco, Rockville, MD, USA) supplied with 10% fetal bovine serum (FBS, Gibco, USA) and 1% streptomycin-penicillin (Sigma-Aldrich, St. Louis, MO, USA) at 37 °C with 5% carbon dioxide (CO_2).

MTT assay

A375 and A2058 cells were seeded into 96-well plates with an inoculation density of 5×10^4 cells/well and maintained at 37 °C in 5% CO_2 for 24 h. Subsequently, cells were incubated with 0, 5, 10, 20, 50, and 100 μM chrysophanol (dissolved in DMSO, Solarbio, Beijing, China) for 24 and 48 h. Then, cells were hatched with 10 μl MTT solution (Sigma-Aldrich) for 4 h. Next, the culture supernatant was discarded, and 100 μl DMSO was added to each well to dissolve the crystals. The absorbance was analyzed at 570 nm using the microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

Colony formation assay

A375 and A2058 cells were inoculated into 6-well plates in a density of 1×10^6 cells/well. Cells were hatched with 0, 5, 10, 20, 50, and 100 μM chrysophanol and maintained for 2 weeks at 37 °C with replacement of the media every 7 days. The clones were immobilized with 4% paraformaldehyde (Solarbio, Philippines) for 20 min and stained with 0.1% crystal violet (Sigma-Aldrich) for 10 min. The clone numbers were counted manually and captured with a digital camera (Canon, Tokoyo, Japan).

Apoptosis analysis

The apoptosis of A375 and A2058 cells was determined by flow cytometry assay. Briefly, A375 and A2058 cells were plated into 24-well plates with an inoculation density of 2.5×10^5 cells/well for cultivation overnight. Then, cells were administrated with 0, 5, 10, 20, 50, and 100 μM chrysophanol for 24 h. Subsequently, cells were collected, washed by phosphate buffer saline (PBS) (Solarbio), and resuspended by 0.5 ml of bind buffer, and stained with 5 μl propidium iodide (PI) (Thermo Fisher Scientific) and 5 μl Annexin V/FITC (Thermo Fisher Scientific) for 15 min at room temperature. The cell apoptotic rate was evaluated by a FACScan flow cytometry using CellQuest software (BD Biosciences, NJ, USA) as shown by the summation of the ratio in quadrants 2 (Q2) and Q3.

Cell scratch assay

A375 and A2058 cells were inoculated into 6-well plates with a density of 1×10^6 cells/well and cultured at 37 °C until the confluence reached at 95%. Subsequently, cells were incubated with 0, 5, 10, 20, 50, and 100 μM chrysophanol for 24 h. The scratch wound was achieved by 200-μl pipette tips. Images were captured by an inverted microscope (Olympus, Tokyo, Japan) at 0 and 24 h.

Transwell assay

The invasion of A375 and A2058 cells was evaluated using the transwell inserts (24-well, 8-μm pore; Corning Costar, Cambridge, MA, USA). The transwell lower chambers were covered with 600 μl of DMEM supplied with 10% FBS. A375 and A2058 cells were collected and resuspended with serum-free DMEM and then plated into the transwell upper chambers filled with 0.1% Matrigel (BD Biosciences) with an inoculation density of 1×10^5 cells/well. Subsequently, diverse concentrations of chrysophanol (0, 5, 10, 20, 50, and 100 μM) were hatched with A375 and A2058 cells for 24 h. Cells were immobilized with 4% paraformaldehyde and then stained with 0.1% crystal violet. Images were photographed by an inverted microscope (Olympus, Japan).

Cell cycle analysis

A375 and A2058 cells were cultured into 6-well plates with an inoculation density of 1×10^6 cells/well and incubated with 0, 5, 10, 20, 50, and 100 μM chrysophanol for 24 h. Cells were collected, rinsed, and resuspended with 250 μl ice-cold PBS. Subsequently, cells were immobilized with 750 μl ice-cold ethanol (100%, Solarbio) for 8 h, and rinsed with PBS. Next, cells were administrated with 50 μg/ml RNase (Thermo Fisher Scientific) and PI for 30 min. The cell cycle was assessed by a FACScan flow cytometry using CellQuest software (BD Biosciences).

Western blotting

The harvested cells were disrupted through RIPA lysis buffer (Sangon Biotech, Shanghai, China), and cellular proteins were collected via centrifugation. The protein concentration of lysate was quantified with the BCA kit (Bio-Rad, Richmond, CA, USA). The protein samples were resolved using 10% SDS-PAGE and subsequently electro-blotted onto PVDF membranes. The membrane sample was blocked with 3% bovine serum albumin (BSA, Sangon Biotech) for 1 h at room temperature and incubated with corresponding primary antibodies at 4 °C overnight. Appropriate secondary antibodies were supplied and incubated for 1 h at 37 °C. All bands were visualized with Amersham ECL Kit (GE Healthcare, Chalfont St. Giles, UK). The primary antibodies utilized in this study were from Cell Signaling Technology, Inc. (Danvers, MA, USA) as follows: Bax

(R 1:1000; 2774), Bcl-2 (M 1:1000; 15071), Cleaved caspase-3 (R 1:1000; 9661), Cyclin D1 (R 1:1000; 2922), CDK4 (R 1:1000; 12790), AKT (R 1:1000; 9272), phospho-Akt (p-AKT; R 1:1000; 9271), ERK1/2 (R 1:1000; 9102), p-ERK1/2 (R 1:1000; 9101), JNK (R 1:1000; 9252), p-JNK (R 1:1000; 9251), p38 (R 1:1000; 9212), and p-p38 (R 1:1000; 9211).

Statistical analysis

All statistical analysis was executed by the SPSS 20.0 software (IBM, Armonk, New York, USA). Data were presented as mean ± standard deviation (SD), and each individual experiment was repeated for at least 3 times. Statistical differences were determined by the one-way analysis of variance (ANOVA) followed by Post Hoc Bonferroni test. $p < 0.05$ was considered significant difference.

RESULTS

Chrysophanol inhibited the proliferation of A375 and A2058 cells

To assess the effects of chrysophanol (Fig. 1A) on the proliferation of melanoma cell, 0, 5, 10, 20, 50, and 100 μM chrysophanol was incubated with A375 or A2058 cells. MTT results showed that the viabilities of A375 and A2058 cells were markedly reduced by administration of 100 μM chrysophanol for 24 h, while treatment of 20, 50, and 100 μM chrysophanol for 48 h prominently reduced the viabilities of A375 and A2058 cells (Fig. 1B). Also, the colony number of A375 and A2058 cells were decreased significantly in a dose-dependent manner by chrysophanol treatment (Fig. 1C,D). Therefore, these results indicated that chrysophanol restrained the proliferation of A375 and A2058 cells.

Chrysophanol promoted the apoptosis of A375 and A2058 cells

The effect of chrysophanol on the apoptosis of A375 and A2058 cells was determined by flow cytometry assay. Chrysophanol observably enhanced the apoptotic rates of A375 and A2058 cells in a dose-dependent manner (Fig. 2A). Besides, chrysophanol also notably elevated the relative protein expression levels of Bax and Cleaved caspase-3 in A375 and A2058 cells in a dose-dependent manner (Fig. 2B). On the contrary, the relative protein expression level of Bcl-2 in A375 and A2058 cells was markedly suppressed by chrysophanol (20, 50, and 100 μM) treatment (Fig. 2B). Thus, these findings illustrated that chrysophanol facilitated the apoptosis of A375 and A2058 cells.

Chrysophanol restrained the migration and invasion of A375 and A2058 cells

Then, cell scratch and Transwell assays were used to determine the effect of chrysophanol on the migration and invasion of A375 and A2058 cells. The relative wound width of A375 and A2058 cells was prominently increased by chrysophanol treatment in a dose-dependent manner (Fig. 3A,B). Additionally, chrysophanol significantly repressed the invasive number of A375 and A2058 cells in a dose-dependent way (Fig. 3C,D). Briefly, chrysophanol suppressed the migration and invasion of A375 and A2058 cells.

Chrysophanol triggered G0/G1 phase arrest of A375 and A2058 cells

Next, flow cytometry assay was utilized to analyze the effect of chrysophanol on the cell cycle of A375 and A2058 cells. Chrysophanol treatment observably elevated the ratio of G0/G1 phase in both A375 and A2058 cells in a dose-dependent manner (Fig. 4A). Moreover, the relative protein levels of Cyclin D1 and CDK4 were notably declined by chrysophanol in a dose-dependent way (Fig. 4B). Taken together, these results elucidated that chrysophanol triggered G0/G1 phase arrest of A375 and A2058 cells.

Chrysophanol regulated the AKT and MAPK signaling pathway in A375 and A2058 cells

To further explore the potential molecular mechanism underlying the effect of chrysophanol on A375 and A2058 cells, the relative protein levels related to the AKT and MAPK signaling pathway were examined via western blotting. The results showed that the relative protein expressions of p-AKT/AKT, p-ERK1/2/ERK1/2, p-JNK/JNK were prominently reduced, while the relative expressions of p-38/38 were significantly enhanced by chrysophanol in a dose-dependent way in both A375 and A2058 cells (Fig. 5). Therefore, these results illuminated that chrysophanol modulated the AKT and MAPK signaling pathway in A375 and A2058 cells.

DISCUSSION

Melanoma, especially CM, is a highly heterogeneous and invasive tumor with poor prognosis due to its resistance to radiotherapy, chemotherapy, and immunotherapy [16]. Chrysophanol, one of the traditional Chinese medicine (TCM), has anti-carcinogenic role in various tumors [6]. In the present study, the anti-carcinogenic effect of chrysophanol was elucidated in both A375 and A2058 cells *in vitro*. The results revealed that chrysophanol induced cell apoptosis and suppressed cell invasion by regulating AKT and MAPK signaling pathway in both A375 and A2058 cells in a dose-dependent manner.

TCM has been employed for the treatment of melanoma such as *Guizhi Fuling* pills [17], *Lycorine hydrochloride* [18], and *Wedelolactone* [19]. Chrysophanol belongs to anthraquinone family that also contains rhein, emodin, and physcion [20]. The anti-carcinogenic activities of chrysophanol were also elaborated in diverse tumors, including colorectal can-

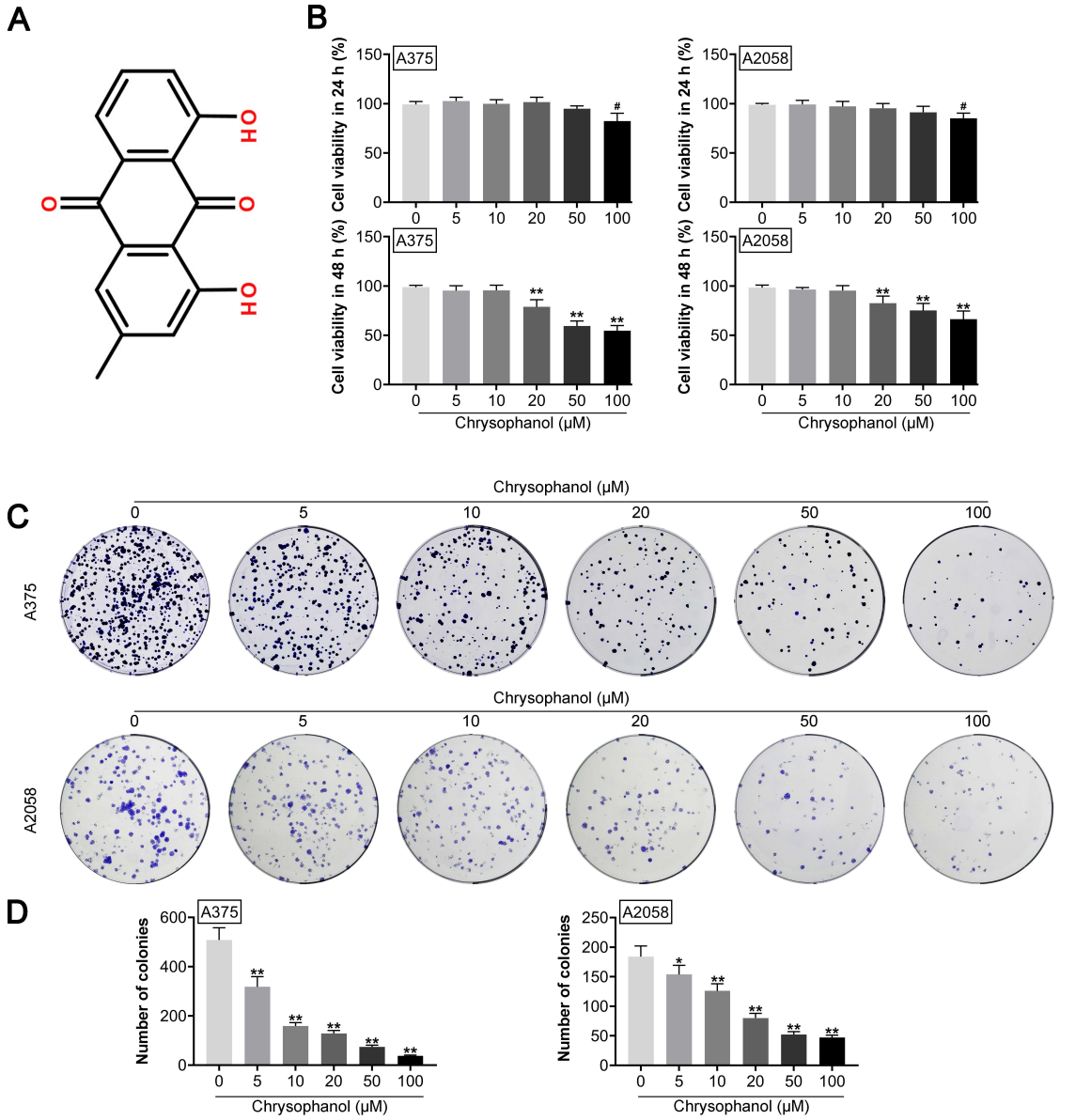

Fig. 1 Chrysophanol inhibited the proliferation of A375 and A2058 cells. Cells were seeded into 96-well plates (5×10^4 cells/well) and maintained at 37 °C in 5% CO_2 for 24 h. (A) Chrysophanol structure. (B) Cell viability after incubated with 0–100 μM chrysophanol for 24 and 48 h. $^{\#}$ $p < 0.05$ vs. Chrysophanol (0 μM) for 24 h; ** $p < 0.01$ vs. Chrysophanol (0 μM) for 48 h. (C and D) Cell proliferation after treated with 0–100 μM chrysophanol for 2 weeks at 37 °C. * $p < 0.05$ and ** $p < 0.01$ vs. Chrysophanol (0 μM).

cer [21] and oral cancer [22]. Moreover, Liu et al [23] reported that emodin inhibits cell proliferation, migration, and invasion with promoted apoptotic of B16F10 and A375 melanoma cells. Similar anti-cancer role of emodin and physcion was also observed in A375 melanoma cells [24]. Furthermore, Zhang et al [15] demonstrated the anti-proliferative role of chrysophanol in melanoma A375 cell line; however, its specific molecular mechanisms still need further investigation.

Here, chrysophanol notably suppressed the proliferation, migration, and invasion of A375 and A2058 cells in a dose-dependent manner. Melanoma progression is closely related to cell proliferation, migration, and invasion [25]. Based on the fact that 20, 50, and 100 μM chrysophanol prominently reduced the

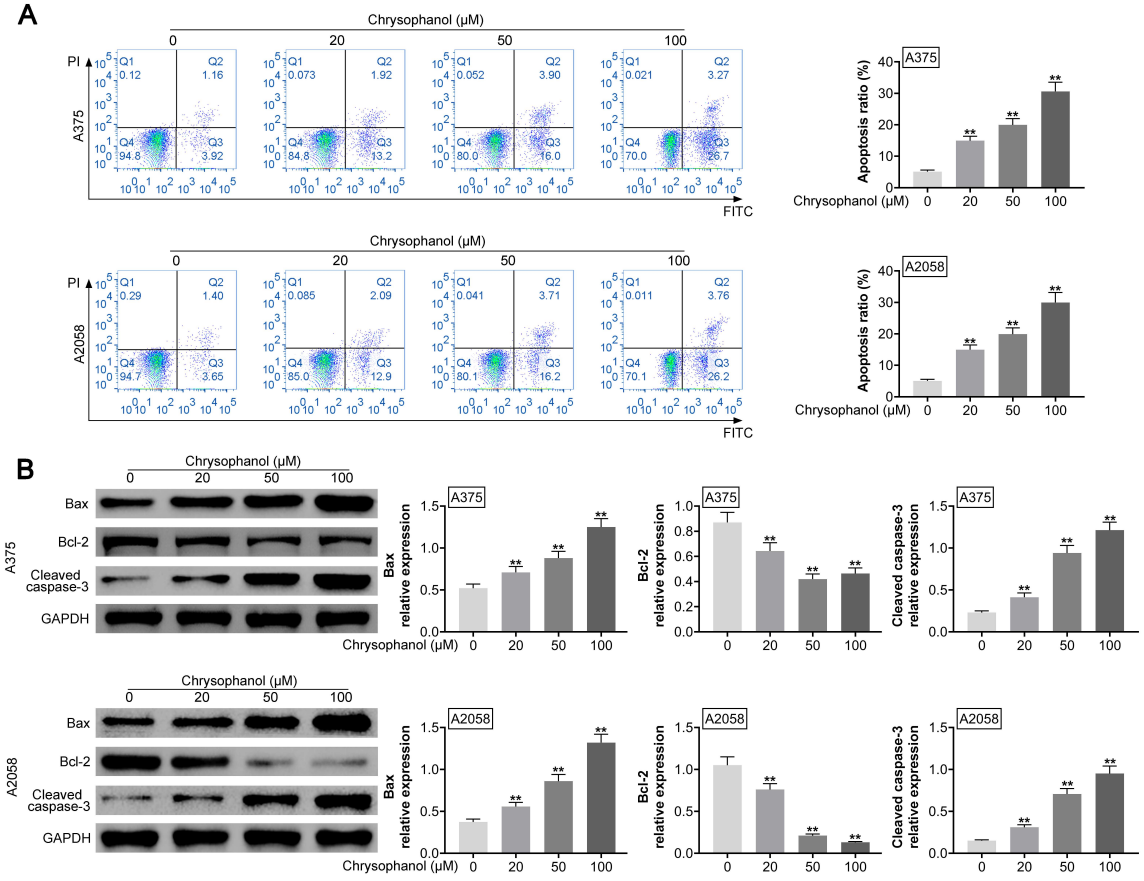

Fig. 2 Chrysophanol promoted the apoptosis of A375 and A2058 cells. Cells were plated into 24-well plates (2.5×10^5 cells/well) for cultivation overnight at 37 °C in 5% CO_2. (A) Cell apoptotic rate after administrated with 0–100 μM chrysophanol for 24 h and then stained with PI and Annexin V/FITC for 15 min at room temperature. The cell apoptotic rate was shown by the summation of ratio in Q2 and Q3. (B) The relative protein levels of Bax, Bcl-2, and Cleaved caspase-3 in cells after treated with 0–100 μM chrysophanol for 24 h. Data were presented after being normalized to GAPDH. The relative intensity of protein was shown as a bar graph. * $p < 0.05$, ** $p < 0.01$ vs. Chrysophanol (0 μM).

viabilities of A375 and A2058 cells for 48 h, these concentrations of chrysophanol were employed for subsequent investigations. Also, the colony formation assay exhibited that the proliferation of A375 and A2058 cells decreased significantly in related to doses of chrysophanol. It is well-known that the modulation of cell cycle, an important factor of proliferation, strongly affects cell growth [26]. In the present study, chrysophanol induced the G0/G1 phase arrest of A375 and A2058 cells in a dose-dependent way. Cyclin D1 mainly accelerates the transformation of cells from G1 phase to S phase, and cyclin D1 can bind to CDK4 to promote the process of S phase gene synthesis, which exert a significant role in cell cycle progression [27]. An example from the previous study showed that ginkgolic acid, an alkylphenol from *Ginkgo biloba* L., could inhibit the proliferation of hepatocellular carcinoma cells by inducing G0/G1 cell cycle arrest [28]. Hence, Cyclin D1 and CDK4 were demonstrated to exert vital roles in cell proliferation in a variety of tumors [29]. Similar to the previous study showing that chrysophanol downregulated the level of Cyclin D1 in breast cancer cells [30], the relative protein levels of Cyclin D1 and CDK4 were notably declined by 20, 50, and 100 μM chrysophanol treatment in the present study, indicating that chrysophanol suppressed the proliferation of A375 and A2058 cell lines in related to its concentration. Additionally, chrysophanol also dampened the migration and invasion of A375 and A2058 cells in a dose-dependent manner according to the cell scratch assay and Transwell assay, respectively. Based on these results, it is thus concluded that chrysophanol observably restrained the proliferation, migration, and invasion of A375 and A2058 cells.

Apoptosis is one of the cell death mechanisms that is associated with multifarious processes [31].

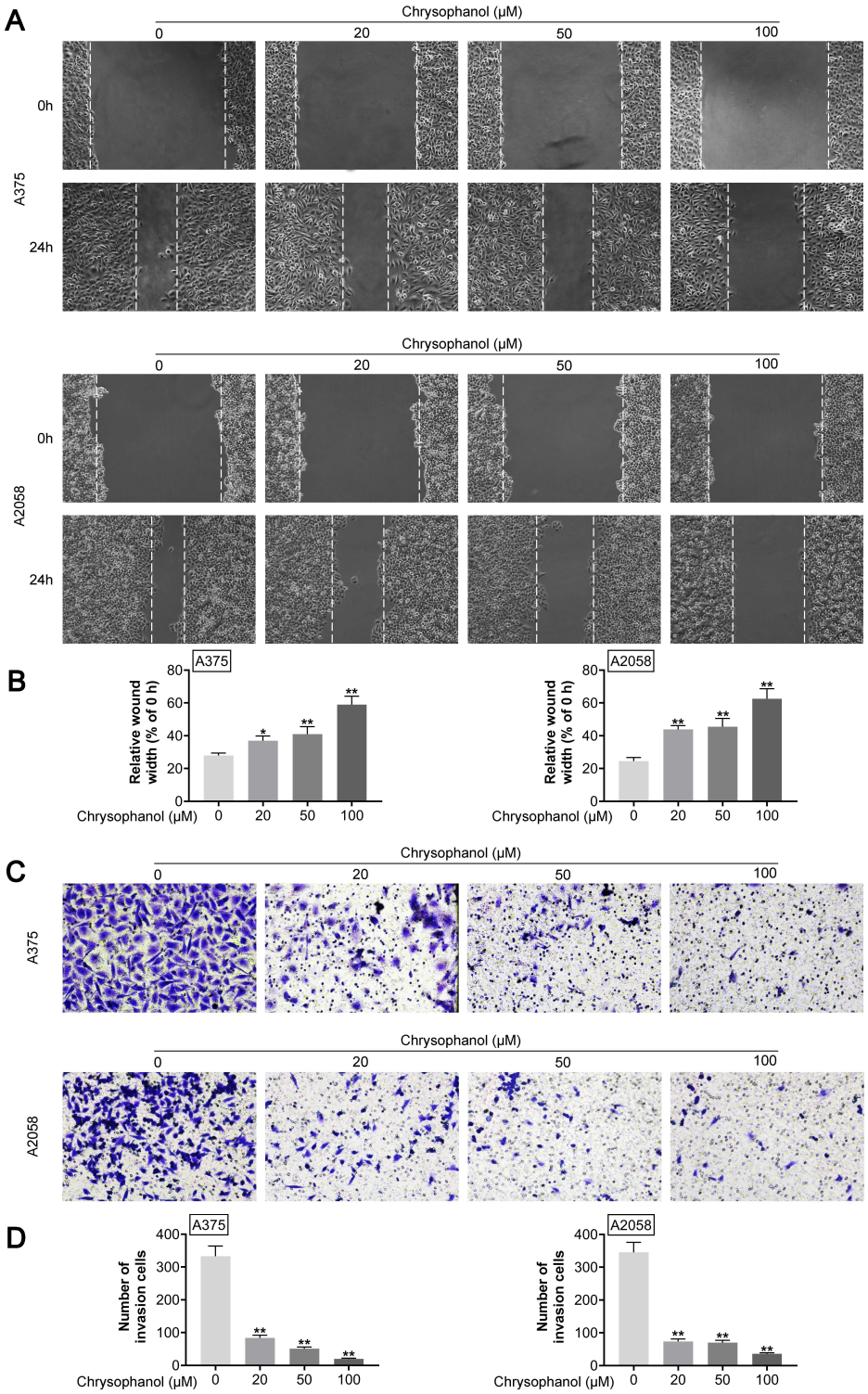

Fig. 3 Chrysophanol suppressed the migration and invasion of A375 and A2058 cells. Cells were plated into 6-well plates (1×10^6 cells/well) and cultured at 37 °C overnight. (A and B) The relative wound width of cells after incubated with 0–100 μM chrysophanol for 24 h. The images were captured by an inverted microscope at 0 and 24 h. (C and D) Cell invasive number after incubated with 0–100 μM chrysophanol for 24 h. Cells were immobilized with 4% paraformaldehyde and then stained with 0.1% crystal violet. * $p < 0.05$ and ** $p < 0.01$ vs. Chrysophanol (0 μM).

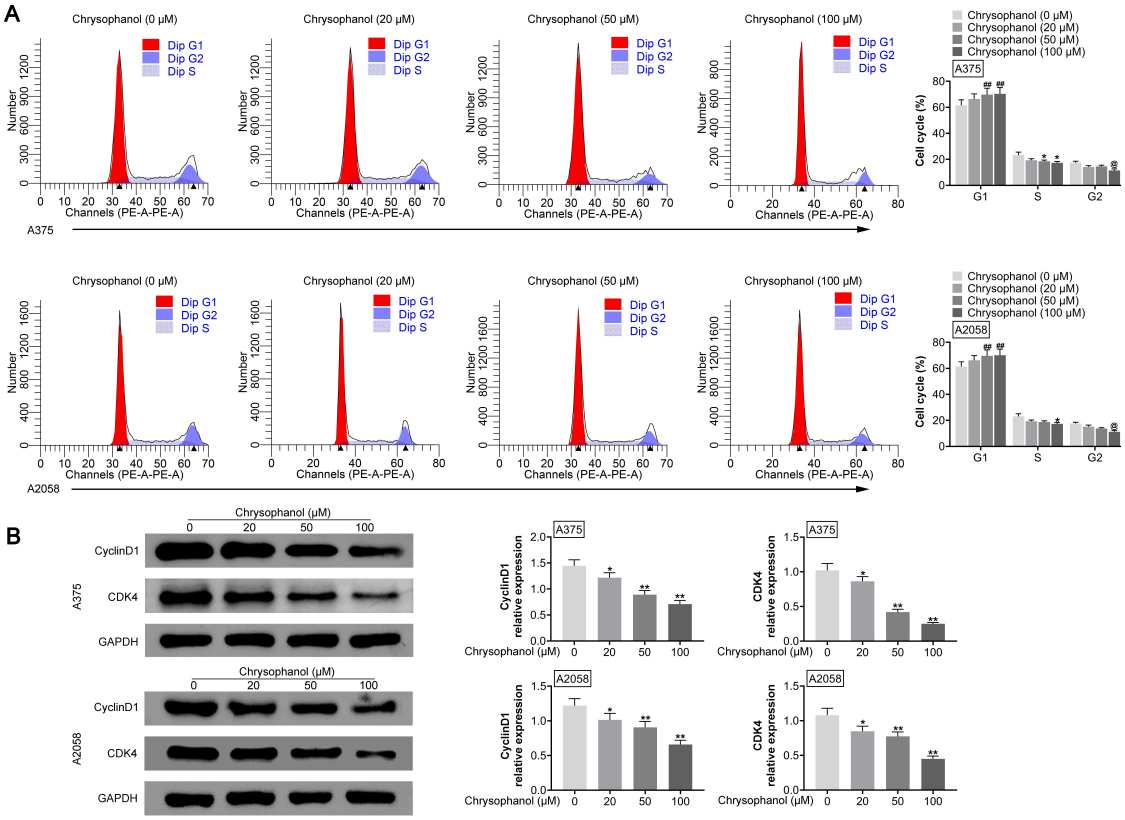

Fig. 4 Chrysophanol triggered G0/G1 phase arrest of A375 and A2058 cells. Cells were cultured into 6-well plates (1×10^6 cells/well) at 37 °C overnight. (A) Cell cycle ratio after incubated with 0–100 μM chrysophanol for 24 h and treated with 50 μg/ml RNase and PI for 30 min. $^{\#\#}p < 0.01$ vs. Chrysophanol (0 μM) for G1; $^{*}p < 0.05$ vs. Chrysophanol (0 μM) for S; $^{@}p < 0.05$ vs. Chrysophanol (0 μM) for G2. (B) The relative protein levels of Cyclin D1 and CDK4 of cells after incubated with 0–100 μM chrysophanol for 24 h. Data were expressed after being normalized to GAPDH. The relative intensity of proteins was shown as a bar graph. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. Chrysophanol (0 μM).

Bax, cleaved-caspase 3, and Bcl-2 are crucial components of apoptosis [32]. Among them, Bcl-2 (an anti-apoptotic gene) and Bax (a pro-apoptotic gene) are two crucial proteins of Bcl-2 family, which regulate cell apoptosis [33]. Cleaved-caspase 3, an activated form of caspase 3, can modulate the diverse phases in the apoptotic pathway [34]. Moreover, the simulative role of chrysophanol in cell apoptosis has been documented in multiple tumors, including breast cancer [30], choriocarcinoma [14], and cervical cancer [35]. Consistent with these findings, our results also exhibited that chrysophanol observably enhanced the apoptotic rate and increased the relative protein expression levels of Bax and cleaved caspase-3, accompanied with the reduced relative protein level of Bcl-2 in both A375 and A2058 cells in a dose-dependent manner. Therefore, these results illustrated that chrysophanol facilitated the apoptosis of A375 and A2058 cells.

AKT and MAPK signaling mediated by ERK, JNK, and p-38 are two significant intracellular signal transduction pathways, which are tightly involved in the tumor development and progression [36]. Their important roles in melanoma were reported in previous studies. For instance, bee venom and its melittin notably inhibited the relative expression levels of p-AKT, p-ERK, and p-p38 in A375SM melanoma cells [37]. Oxyfadichalcone C repressed the cell growth and metastasis of melanoma A375 cells through inhibition of PI3K/AKT and MAPK/ERK signaling pathways [38]. Apigenin prominently reduced the protein levels of p-AKT and p-p38, while promoted the protein levels of p-ERK and p-JNK in A375SM cells [39]. Moreover, multiple pharmacological effects of chrysophanol were elaborated to be associated with PI3K and MAPK signaling pathways [40]. In line with these studies, our results also showed that the relative expressions of p-AKT/AKT, p-ERK1/2/ERK1/2, and p-JNK/JNK were notably decreased, accompanied with the enhanced

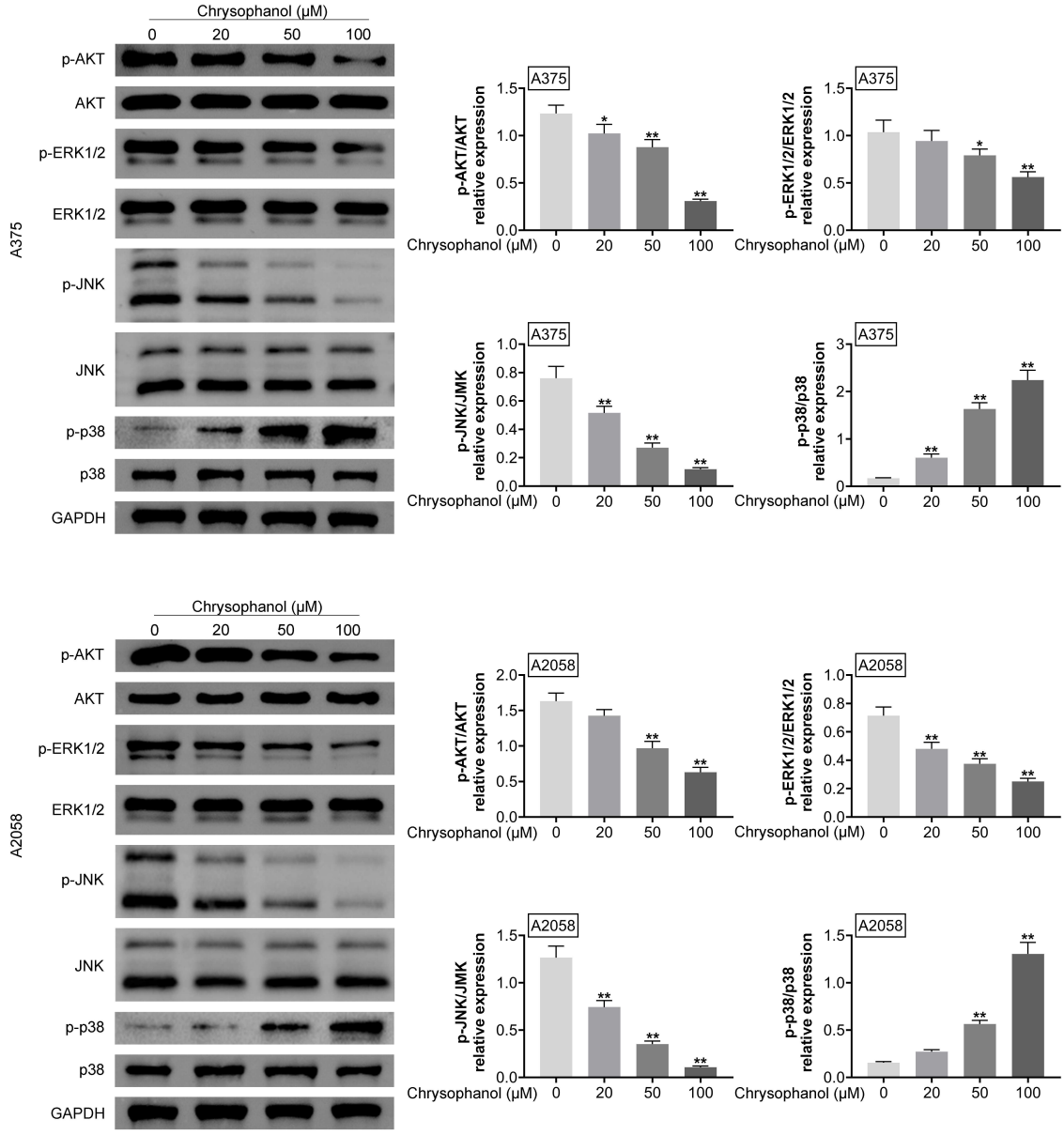

Fig. 5 Chrysophanol modulated the AKT and MAPK signaling pathways in A375 and A2058 cells. Cells were cultured into 6-well plates (1×10^6 cells/well) at 37 °C overnight. The relative protein levels of p-AKT, AKT, p-ERK1/2, ERK1/2, p-JNK, JNK, p-p-38, and p-38 of cells were analyzed after incubated with 0–100 μM chrysophanol for 24 h. Data were shown after being normalized to GAPDH. The relative intensity of protein was shown as a bar graph. * $p < 0.05$ and ** $p < 0.01$ vs. Chrysophanol (0 μM) for A375 cells; * $p < 0.05$ and ** $p < 0.01$ vs. Chrysophanol (0 μM) for A2058 cells.

relative expression of p-38/38 by 20, 50, and 100 μM chrysophanol treatment. Taken together, these results elucidated that chrysophanol regulated the AKT and MAPK signaling pathway in A375 and A2058 cells.

In conclusion, it is illuminated that chrysophanol promoted cell apoptosis and restrained cell invasion via modulating AKT and MAPK signaling pathway in A375 and A2058 cells in a dose-dependent manner. Nonetheless, several limitations of the present study should be addressed: (1) Although the exact effect of chrysophanol on melanoma cell lines was elaborated according to a series of assays, the *in vivo* role needs further investigations; (2) Plenty of researches have revealed the important role of AKT and MAPK signaling

pathway in the cell proliferation, migration, invasion, and apoptosis in various tumors, and therefore the effect of chrysophanol on the cell proliferation, migration, invasion, and apoptosis in melanoma also requires more attentions after blocking or interference of AKT and MAPK signaling pathway. In brief, our results support a better understanding of potential molecular mechanism and contribute to the clinical development of melanoma.

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