Regular paper

Neogambogic acid induces apoptosis of melanoma B16 cells *via* the PI3K/Akt/mTOR signaling pathway

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Background: Neogambogic acid, as one of the main components of gamboge, has obvious antitumor effects. **Objective**: To explore the mechanism by which neogambogic acid induces melanoma B16 cell apoptosis. **Methods**: Melanoma B16 cells were treated with different concentrations of neogambogic acid (0, 1.5, 3.0, 6.0 μM). The proliferation inhibition rate was measured by MTT assay. Cell morphology was assessed by inverted microscope. Cell migration and invasion were tested by Transwell assay. The apoptosis rate and cell cycle were detected by flow cytometry. The level of proteins related to the PI3K/Akt/mTOR signaling pathway was measured by Western blot. **Results**: The proliferation inhibition rate of B16 cells significantly increased with rising neogambogic acid concentration ($P<0.05$). The invasive and migration capacities of B16 cells decreased significantly after treatment with neogambogic acid ($P<0.05$). The apoptosis rate also increased with the rising concentration of neogambogic acid. After 24 h of treatment, the percentage of cells in the G0/G1 phase increased with the neogambogic acid concentration, whereas those of cells in S and G2/M phases decreased. With increasing concentration of neogambogic acid, the level of p-PI3K, p-Akt and p-mTOR proteins was reduced in a time-dependent manner, but for PI3K and Akt proteins it remained basically unchanged. **Conclusion**: Neogambogic acid can inhibit the proliferation, invasion and migration of melanoma B16 cells and induce their apoptosis, which may be regulated *via* the PI3K/Akt/mTOR signaling pathway.

Key words: apoptosis, invasion, melanoma, metastasis, neogambogic acid, proliferation

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Abbreviations: FBS, Fetal bovine serum; PI, propidium iodide

INTRODUCTION

Melanoma occurs mostly in the skin, accompanied by the mucosae of the digestive system, reproductive system, eyes, pia mater and choroid (Hayward *et al.*, 2017). Its incidence rates vary among races, regions and ethnicities (Shen *et al.*, 2017). At present, the Chinese population is also endangered by malignant melanoma (Zhu *et al.*, 2016).

Malignant melanoma is formed by the malignant transformation of melanocytes and capable of producing melanin, as a serious threat to life . It can invade tissues, continuously proliferate and escape immune surveillance (Slominski *et al.*, 2004). Until now, there is still no effective prevention or treatment method. Surgery is usually performed at the early stage, as soon as possible after diagnosis. However, patients are prone to relapse after surgery, requiring chemotherapy based on dacarbazine, temozolomide, carboplatin and paclitaxel. Although the effects of multi-agent chemotherapy are slightly superior to those of single-agent chemotherapy, the cure rate remains low. Radiotherapy works well for patients who had incomplete surgical resection and refuse to or cannot receive surgery. The other therapies include cellular immunotherapy, cytokine therapy, monoclonal antibody therapy, etc. For the treatment of advanced malignant melanoma, palliative surgery and chemotherapy are still mainly employed in clinical practice, accompanied by the use of interferon-a1b, CIK and DC-CIK. Nevertheless, treatment outcomes remain unsatisfactory. Therefore, it is urgent to find a new treatment method for malignant melanoma (Slominski & Carlson, 2014).

Traditional Chinese medicine drugs have unique advantages in antitumor treatment, and among them, eligible drugs for malignant melanoma therapy may be found. Researchers have endeavored to screen antitumor agents from plants. The dry resin (gamboge) secreted by plants of the *Clusiaceae* family is well-documented to be sour and toxic, being capable of destroying gangrenous skin ulcers, eliminating thrombi and dispersing nodules. It has mainly been used to treat ulcers, swelling, chronic dermatitis, sore, bruises, traumatic bleeding and burns (Wang *et al.*, 2018). Neogambogic acid (Fig. 1), as one of the main components of gamboge, has evident antitumor effects (Sun *et al.*, 2018). Therefore, we herein intended to study the possible mechanism by which neogambogic acid induces the apoptosis of mouse skin melanoma B16 cells.
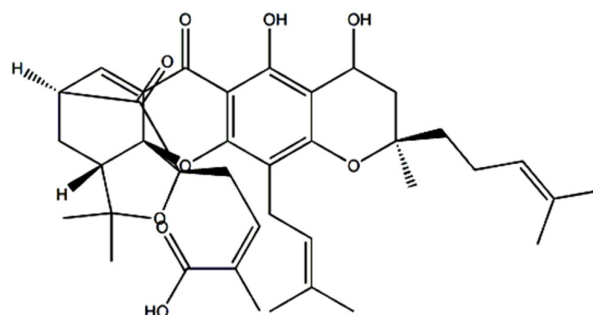

Figure 1. Chemical structure of neogambogic acid.

MATERIALS AND METHODS

Cells, reagents and apparatus. Mouse melanoma B16 cells were obtained from ATCC (USA). Neogambogic acid was purchased from Shanghai Hewu Biotechnology Co., Ltd. (China). RPMI 1640 medium was bought from Gibco (USA). Fetal bovine serum (FBS) and electrochemiluminescence reagent were provided by Thermo Fisher Scientific (USA). Trypsin was obtained from Ameresco (USA). Akt, GAPDH, PI3K and p-PI3K antibodies were purchased from Santa Cruz Biotechnology (USA). P-Akt (Ser473), PTEN and p-mTOR antibodies were bought from Cell Signaling Technology (USA).

Cell culture. B16 cells were seeded into culture flasks with RPMI 1640 medium containing 10% FBS, 100 U/mL penicillin and 100 U/mL streptomycin, and incubated at 37°C with 5% CO_2 and 100% humidity. After cell adherence, the culture medium was refreshed every other day. At the confluence of about 90%, the cells were digested with trypsin and subcultured every three days. Cells in the logarithmic growth phase were used for subsequent experiments.

Cell grouping and treatment. B16 cells were treated with neogambogic acid at 0, 1.5, 3.0 and 6.0 μM. Time points were set at 24, 48 and 72 h.

Detection of B16 cell proliferation by MTT assay. B16 cells were detached, collected and inoculated into a sterile 96-well plate at a density of $6×10^4$/ml, 100 μL per well. Four replicate wells and a blank control group were set. The cells were cultured overnight until adherence. The cells were treated with different concentrations of neogambogic acid solutions (0, 1.5, 3.0, 6.0 μM) in an incubator for 24, 48 and 72 h. Four hours before each timepoint the medium was carefully removed, and 20 μL of MTT solution was added to each well to continue culture for 4 h at 37°C. Then, 150 μL of DMSO was added to each well and shaken for 20 min. The absorbance (A) of each well was measured by a microplate reader at 570 nm, and the experiment was repeated three times. Inhibition rate = $(A_{\text{negative control}} - A_{\text{experimental group}})$/$A_{\text{negative control}}$ × 100%.

Assessment of B16 cell morphology by inverted microscope. B16 cells were trypsinized, collected and inoculated into culture flasks at a density of $2×10^5$/mL, 4 mL per flask. The cells were cultured with 5% CO_2 at 37°C for 24 h. At the confluence of about 70%, the culture medium was discarded, and the cells were thereafter treated with fresh media containing different concentrations of neogambogic acid for 24 h and finally observed under an inverted microscope.

Detection of B16 cell migration and invasion by Transwell assay. **Migration experiment**. Cells were diluted with serum-free RPMI 1640 medium and adjusted to the concentration of $1×10^5$/mL. Subsequently, 200 μL of the cell suspension was added to the Transwell upper chamber coated with Matrigel, and different concentrations of neogambogic acid solutions (0, 1.5, 3.0, 6.0 μM) were added. Then, 500 μL RPMI 1640 medium containing 20% FBS was added to the Transwell lower chamber for 24 h of incubation. The upper chamber was removed and washed twice with pre-cooled PBS, and the cells on the upper surface were gently wiped off. The cells on the membrane were fixed by 4% paraformaldehyde solution for 15 min and stained with crystal violet for 2 h at room temperature. The cells penetrating the membrane were counted by microscopy at high magnification, and those in five randomly selected visual fields were photographed using Image-Pro Plus 6.0 software. The protocol of the invasion experiment was basically the same as that of migration experiment, except Matrigel was not used.

Detection of B16 cell apoptosis by flow cytometry. The cell culture medium was transferred to a 15 ml centrifuge tube, and the adherent cells were washed twice with PBS and digested with 1 ml of trypsin containing EDTA at room temperature for 2 min, which was terminated by adding 2-4 ml of complete medium. The cell culture medium was centrifuged at $1000×g$ for 5 min, and the supernatant was discarded. Afterwards, the cells were collected, resuspended in PBS and counted. The resuspended cells were centrifuged at $1000×g$ for 5 min, and the supernatant was discarded. Then, the cells were resuspended by adding 195 μl of annexin V-FITC buffer. Subsequently, the cells were mixed with 5 μl of annexin V-FITC and 10 μl of propidium iodide (PI) staining solution. Then, the cells were incubated for 15 min at room temperature in dark, during which they were resuspended twice. Finally, cell apoptosis was detected by flow cytometry three times independently.

Detection of B16 cells' cell cycle by flow cytometry. After cells were digested in a 15 ml centrifuge tube, the cell culture medium was centrifuged at $1000×g$ for 5 min, and the supernatant was discarded. Afterwards, the cells were collected, resuspended in 1 ml of pre-cooled PBS and transferred to another 1.5 ml centrifuge tube. The resuspended cells were centrifuged at $1000×g$ for 5 min, and the supernatant was discarded. Subsequently, 1 ml of pre-cooled 70% ethanol solution was added in a way to fully resuspend the cells which were fixed at 4°C for 24 h and centrifuged at $1000×g$ for 5 min, and the supernatant was discarded. The cells were resuspended in 1 ml of pre-cooled PBS and mixed with 600 μl of PI staining solution (20×) and 240 μl of RNase A (50×). The cell precipitate was resuspended by adding 0.5 ml of PI staining solution and incubated at 37°C for 30 min. Flow cytometry was carried out within 24 h after staining. The percentages of cells in G1, S and G2 phases were calculated by FlowJo software (USA). The experiments were carried out three times, and the averages were reported.

Detection of the level of proteins related to the PI3K/Akt/mTOR signaling pathway by Western blot. Cells were trypsinized, centrifuged, inoculated into a 6-well plate at a density of $1×10^5$/well and cultured at 37°C with 5% CO_2. After adherence, the cells were treated with different concentrations of neogambogic acid solutions (0, 1.5, 3.0, 6.0 μM) for 30 min, and the supernatant was discarded. After being washed twice with pre-cooled PBS, the cells were lysed in RIPA lysis buffer for 30 min and centrifuged at $11\,000×g$, 4°C for 10 min to collect the supernatant. After 5% SDS-PAGE, the proteins were electronically transferred onto a polyvinylidene fluoride membrane at 4°C for 1.5 h. Then, the membrane was blocked with 5% skimmed milk for 2 h, incubated with primary antibodies overnight at 4°C, washed in TBST, incubated with horseradish peroxidase-labeled goat anti-rabbit IgG antibody at 37°C for 2 h and color-developed with electrochemiluminescence reagent. Images were acquired by an automatic gel imaging system. Protein levels were measured by using GAPDH as the internal reference.

Statistical analysis. All the data were statistically analyzed in SPSS16.0 software. The normally distributed quantitative data were expressed as mean ± standard deviation. Intergroup comparisons were conducted by the independent t-test. $P<0.05$ was considered statistically significant.
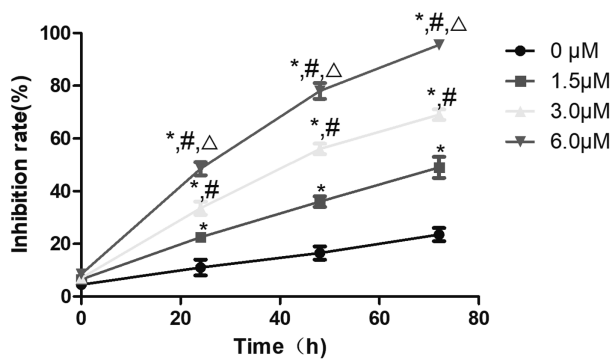
Figure 2. **Effects of neogambogic acid on B16 cell proliferation evaluated by MTT assay.**
*Compared to 0 μM group at the same time point, $P<0.05$; #compared to 1.5 μM group at the same time point, $P<0.05$; Δcompared to 3.0 μM group at the same time point, $P<0.05$.

RESULTS

Effects of neogambogic acid on B16 cell proliferation evaluated by MTT assay

The proliferation inhibition rate of B16 cells significantly increased with rising neogambogic acid concentration ($P<0.05$). With increasing concentration from 0 to 6.0 μmol·L^{-1}, B16 cell proliferation was suppressed dose- and time-dependently (Fig. 2).
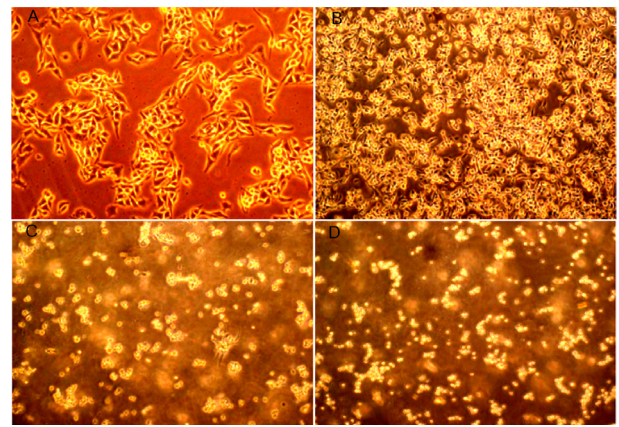

Figure 3. Morphological changes of B16 cells after treatment with different concentrations of neogambogic acid.

Effects of neogambogic acid on B16 cell morphology assessed by inverted microscopy

As assessed under the inverted microscope, untreated B16 cells expanded well and had a uniform cytoplasm. After treatment with 1.5 μM neogambogic acid for 24 h, some cells underwent pyknosis, became round and shrank. Meanwhile, a small number of cells were semi-adherent. After treatment with 3.0 μM neogambogic acid for 24 h, most cells shrank, detached and floated in the culture medium. A small number of cells enlarged, swelled and ruptured. After treatment with 6.0 μM ne-
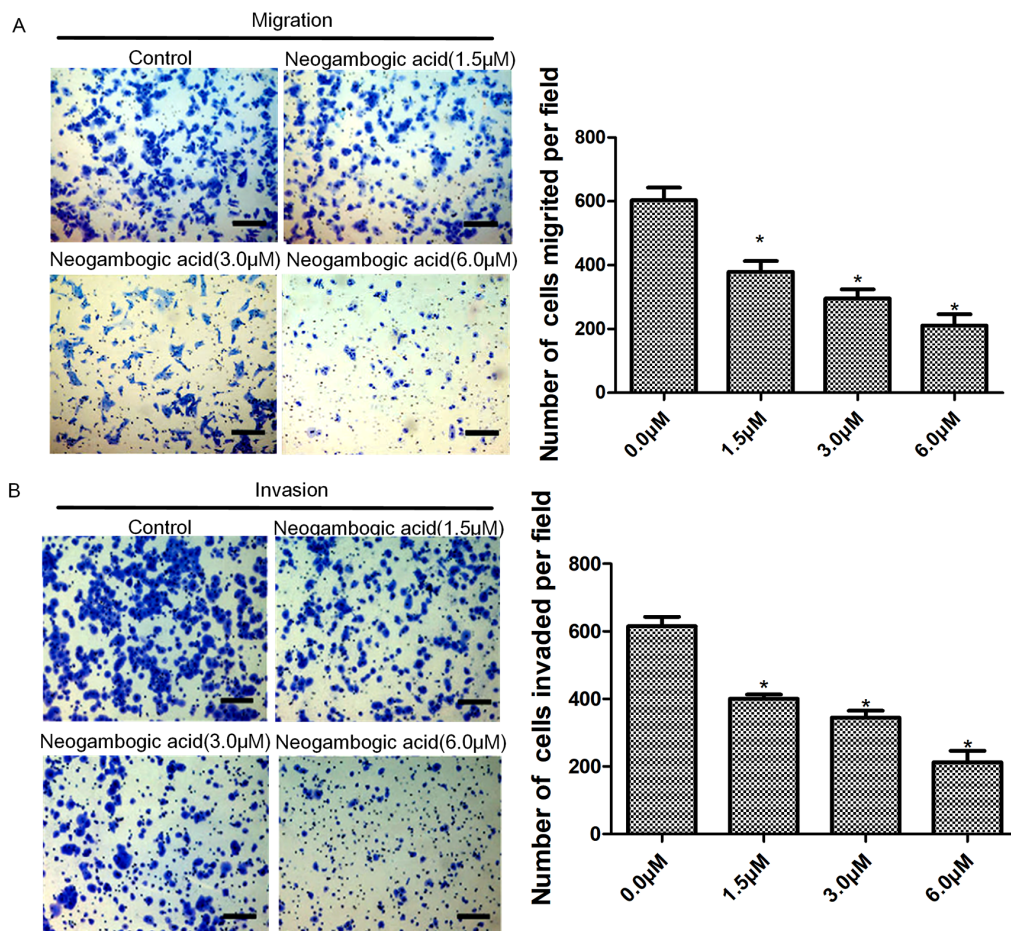

Figure 4. Effects of neogambogic acid on B16 cells migration and invasion.
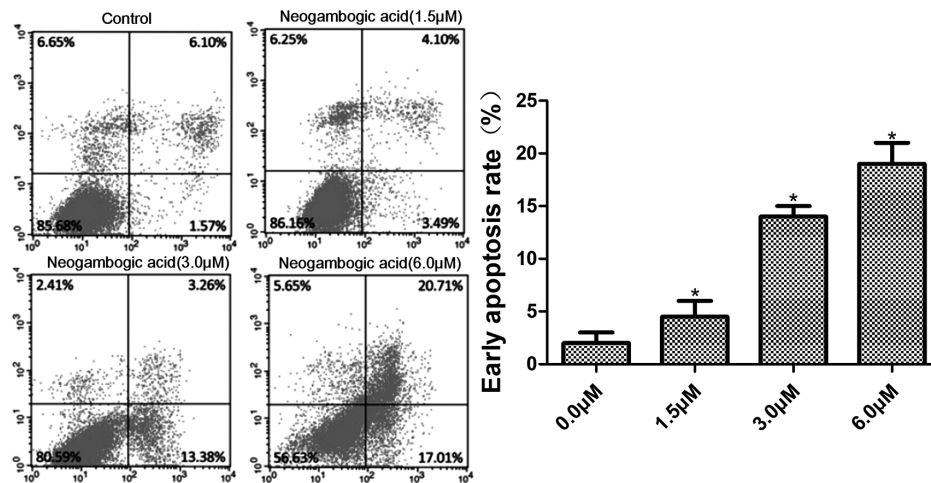*Compared to Control, $P<0.05$.

Figure 5. Effects of neogambogic acid on B16 cells apoptosis.
*Compared to Control, *P*<0.05.

ogambogic acid, most cells were round and non-adherent, floating in the culture medium (Fig. 3).

Effects of neogambogic acid on B16 cells migration and invasion

Transwell assay showed that the invasive and migration capacities of B16 cells decreased significantly after treatment with neogambogic acid (*P*<0.05) (Fig. 4).

Effects of neogambogic acid on B16 cells apoptosis

Flow cytometry revealed that the apoptosis rate of B16 cells markedly increased with rising concentrations of neogambogic acid (Fig. 5).

Effects of neogambogic acid on B16 cells' cell cycle

After 24 h of treatment, the percentage of cells in the G0/G1 phase increased as the neogambogic acid con-

centration rose, whereas those of cells in S and G2/M phases decreased (Fig. 6).

Effects of neogambogic acid on proteins related to the PI3K/Akt/mTOR signaling pathway

With increasing concentration of neogambogic acid, the level of p-PI3K, p-Akt and p-mTOR proteins decreased in a time-dependent manner, but for PI3K and Akt proteins it remained basically unchanged (Fig. 7).

DISCUSSION

Melanoma is a common lethal skin cancer, and its incidence rate worldwide has increased each year (McGettigan, 2014; Lens & Dawes, 2015). A multistep and highly regulated pathway of melanin synthesis is the main function discriminating the malignant and normal melanocytes. Although melanin primarily protects against UV-induced damage, this pigment also modulates epidermal homeo-
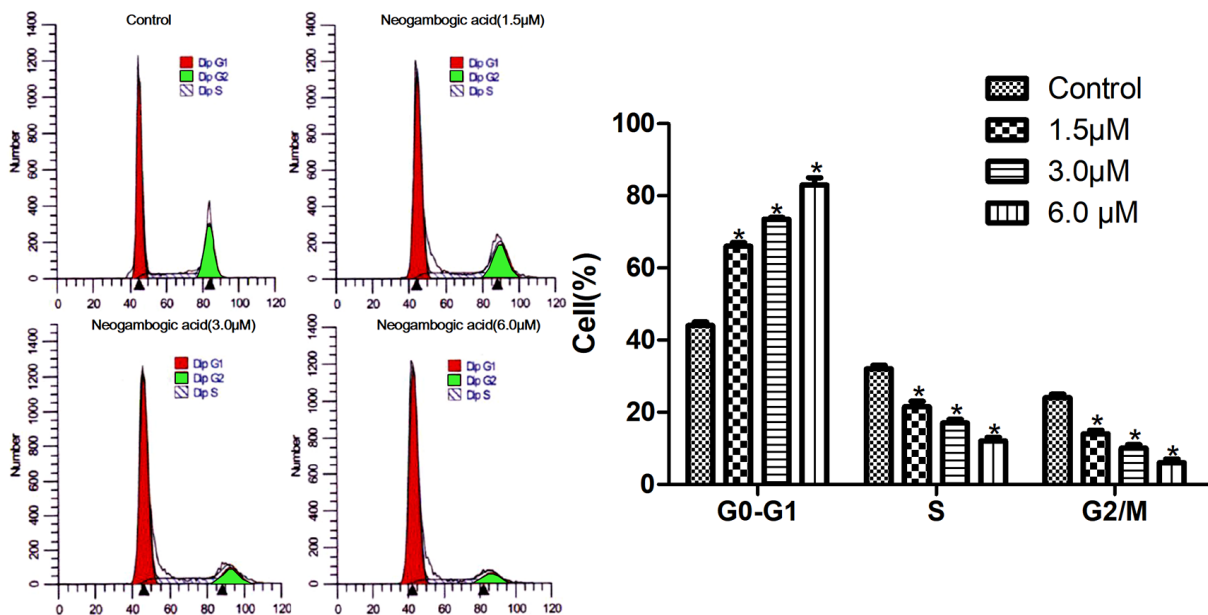

Figure 6. Effects of neogambogic acid on B16 cells' cell cycle.
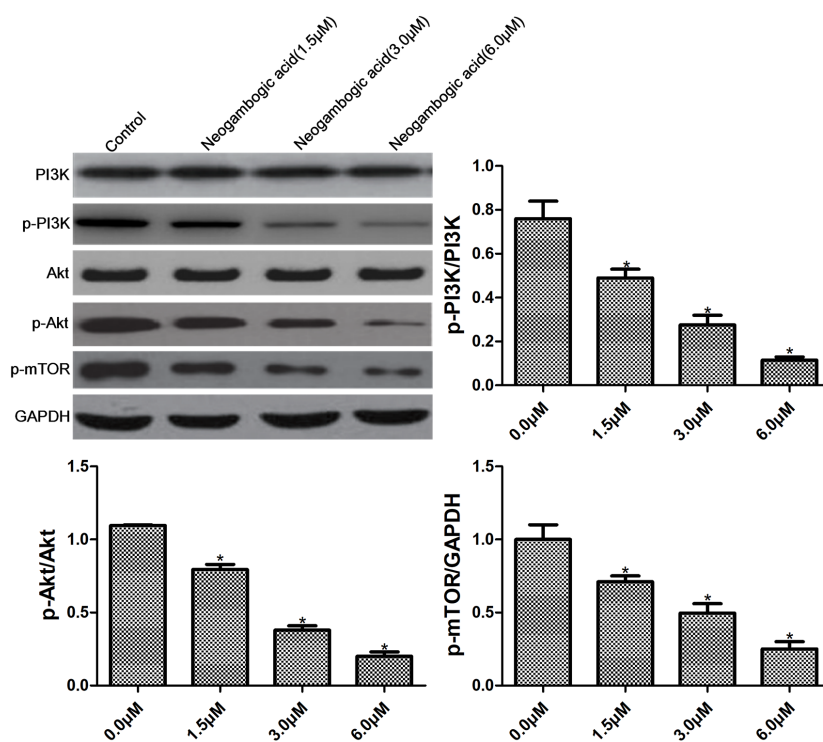*Compared to Control, *P*<0.05.

Figure 7. Effects of neogambogic acid on proteins related to the PI3K/Akt/mTOR signaling pathway.
*Compared to Control, P<0.05.

stasis and thus influences melanoma behaviors (Slominski *et al.*, 2014). As a result of complex interactions between constitutional, genetic and environmental factors, skin melanoma originates from genetically altered or activated epidermal melanocytes (Slominski *et al.*, 2015). Melanoma is one of the most common malignancies among the white population, with a mortality rate exceeded only by that of lung cancer (Slominski *et al.*, 2009). The high mortality rate of melanoma cases is associated with the progression to resistance at stages III and IV. Moreover, the tumor can only be clinically cured by surgery in the radial growth phase (Linette *et al.*, 2005). Nevertheless, once metastasis starts, melanoma becomes resistant to all currently available therapies (Brożyna *et al.*, 2016).

The traditional therapeutic drugs for melanoma include dacarbazine, temozolomide and interleukin 2, as well as FDA-approved targeted drugs such as ipilimumab. However, their use is limited due to short response time, tumor resistance and adverse reactions (Rigel *et al.*, 2010; Menaa, 2013). Therefore, it is urgent to develop new therapeutic strategies and targets for melanoma.

Gamboge has well-established inhibitory effects on the proliferation of various tumor cells (Li *et al.*, 2010; Mei *et al.*, 2014). In 1984, Lu *et al.* (1984) first isolated neogambogic acid from gamboge. Compared to gambogic acid, neogambogic acid has a stronger antitumor effect, lower toxicity, higher stability and broader anticancer spectrum. In this study, the proliferation inhibition rate of B16 cells significantly increased with rising neogambogic acid concentration (*P*<0.05). In addition, the invasive and migration capacities of B16 cells decreased significantly after treatment with neogambogic acid (*P*<0.05).

Apoptosis plays an important role in the onset and progression of tumors and may provide a target for the treatment of tumors (Wang *et al.*, 2014). Herein, the apoptosis rate also increased with the rising concentration of neogambogic acid. Thus, inducing apoptosis by ne-

ogambogic acid may be one of the mechanisms for inhibition of the tumor cell growth and proliferation.

The cell cycle refers to the cyclic process in which eukaryotic cells continue to divide from the end of one mitosis to the end of the next division (Abreu Velez & Howard, 2015). In this study, after 24 h of treatment, the percentage of cells in the G0/G1 phase increased as the neogambogic acid concentration rose, whereas those of cells in S and G2/M phases decreased. Changes in the cell cycle phases distribution after the administration of the compound may be attributed to the variations of cell properties, but further research is needed to explore the underlying mechanisms.

The PI3K/Akt/mTOR signaling pathway is composed of PI3K, Akt and mTOR proteases. Activation of this pathway can inhibit apoptosis induced by a variety of stimuli and promote cell cycle progression, thereby facilitating tumor cell survival and proliferation. It also participates in angiogenesis, as well as the occurrence, development, drug resistance, invasion and metastasis of malignant tumors (Hahne *et al.*, 2017; Yang *et al.*, 2017; Adimonye *et al.*, 2018). With increasing concentration of neogambogic acid, the level of p-PI3K, p-Akt and p-mTOR proteins was reduced in a time-dependent manner, but for PI3K and Akt proteins it remained basically unchanged.

Regardless, this study still had some limitations. Only *in vitro* experiments using mouse melanoma B16 cells were conducted. Further in-depth studies concerning the *in vivo* antitumor effects of neogambogic acid on melanoma and those employing human melanoma cells are ongoing in our group.

CONCLUSION

In summary, neogambogic acid can suppress the proliferation, invasion and migration of melanoma B16 cells

and induce their apoptosis, which may be regulated via the PI3K/Akt/mTOR signaling pathway.

Acknowledgments

Conflicts of interest

There are no conflicts of interest.

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