

Antitumorigenic effect of damnacanthal on melanoma cell viability through p53 and NF- κ B/caspase-3 signaling pathways

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Abstract. Melanoma is highly malignant, particularly prone to metastasizing to the skin. The incidence of melanoma varies markedly between countries, and is relatively low in China. The aim of the present study was to investigate the antitumorigenic effect of damnacanthal on melanoma cells, and its molecular mechanism. MUM-2B cells were treated with 0-20 μ M damnacanthal for 12, 24 and 48 h. *In vitro*, it was demonstrated that damnacanthal inhibited proliferation and promoted apoptosis of melanoma cells in a dose- and time-dependent manner. Damnacanthal treatment increased caspase-3/8 and 9 activity, and promoted B-cell lymphoma 2-associated X protein, tumor protein p53 (p53) and p21 protein expression levels in melanoma cells. Damnacanthal treatment also resulted in downregulated nuclear factor- κ B (NF- κ B), cyclin D and cyclin E protein expression in melanoma cells. In conclusion, the results of the present study demonstrated that the antitumorigenic activity of damnacanthal on melanoma cells is executed via the p53/p21 and NF- κ B/cyclin/ caspase-3 signaling pathways.

Introduction

Melanoma is the most common type of malignant tumor that occurs in the conjunctiva, accounting for between 2 and 5% cases of eye cancer, and between 5 and 7% cases of primary malignant melanoma of the eye (1). The incidence of melanoma is reported to have increased gradually, which may be associated with increased ultraviolet exposure (2). The high degree of tumor malignancy is associated with high recurrence rate and

high incidence of adjacent lymph node and liver metastasis (3). Despite treatment, the local recurrence rate is 62%, and the mortality rate ranges between 18 and 44% (3). Current clinical treatment includes wide excision, which aims to remove the total tumor tissue and avoid recurrence caused by residual cancer cells (4). Common adjuvant treatments include freeze therapy, radiotherapy, chemotherapy, coagulation therapy and biological therapy (4). Gradual and comprehensive treatment methods decrease the recurrence rate to some extent; however, following treatment the 5-year local recurrence rate is between 30 and 50%, the 10-year mortality rate is <30% and the prognosis remains poor (3).

Tumor protein p53 (p53) is the tumor suppressor gene which has been most widely associated with human tumors (5). Under normal circumstances, p53 functions in DNA repair and replication, arresting cell proliferation following DNA damage, which inhibits tumor growth (6). Mutant p53 is more stable than the wild-type gene, and can be identified by immunohistochemistry (7). High expression of p53 protein indicates a mutation of p53. p53 has been demonstrated to serve an important function in the occurrence and development of melanoma (5).

Nuclear factor- κ B (NF- κ B) is an important nuclear transcription factor, involved in the regulation of numerous physiological and pathological events, including mediating inflammation, cell survival, apoptosis and tumor invasion (5). NF- κ B has also been implicated in the association between inflammation and cancer occurrence (5).

Anthraquinone compounds have been identified in Rubiaceae, Polygonaceae and leguminous plants, and their extraction and separation, component analysis and pharmacological effects have been investigated (8). The basic quinone nuclei of the commonly used anticancer drugs, doxorubicin and mitoxantrone, have an anthraquinone structure (8). However, the mechanism of the antitumor effect of anthraquinones remains unclear. Damnacanthal (Fig. 1) is a derivative of *Damnacanthus* (a flowering plant of the Rubiaceae family native to eastern Asia), and has been demonstrated to exhibit potent anticancer properties (9). Previous studies have demonstrated that the compound exhibits cytotoxic activity and anticancer cell proliferation effects, which may result in the apoptosis of cancer cells and an antitumor function (8,10). The aim of the present study was to investigate the

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underlying molecular mechanism of antitumorigenic activity of damnacanthal on melanoma cells.

Materials and methods

Cell culture. The human melanoma cell line MUM-2B was obtained from the Cell Bank of the Chinese Academy of Sciences (Beijing, China) and cultured in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Inc.) in 5% CO₂ at 37°C.

MTT cell viability assay. A total of 1x10⁴ MUM-2B cells were seeded in a 96-well plate for 24 h, then treated with 0, 1, 2.5, 5, 10 or 20 µM damnacanthal for 12, 24 or 48 h. A total of 20 µl MTT (5 mg/ml; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was added to each well prior to incubation for another 4 h at 37°C. The supernatants were aspirated and 150 µl dimethylsulfoxide (Sigma-Aldrich; Merck KGaA) was added to each well to dissolve the formazan crystals for 20 min at 37°C. The optical density (OD) at 490 nm was determined using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

DAPI assay. Apoptosis was analyzed using a DAPI assay (Beyotime Institute of Biotechnology, Haimen, China), according to the manufacturer's protocol. MUM-2B cells were seeded in a 6-well plate at 1x10⁶ cells/well for 24 h. The cells were treated with 2.5, 5 and 10 µM damnacanthal for 24 h, prior to being washed twice in PBS. A concentration of 100 ng/ml DAPI assay reagent was added for 10 min at room temperature in the dark. The MUM-2B cells were then washed twice with PBS and observed using a fluorescence microscope at x400, magnification.

Flow cytometry. Apoptosis was also detected using flow cytometry. MUM-2B cells were seeded in a 6-well plate at 1x10⁶ cells/well for 24 h and treated with 2.5, 5 and 10 µM damnacanthal for 24 h. The cells were then harvested, washed and resuspended in ice-cold PBS. The cells were stained with Annexin V-fluorescein isothiocyanate (50 µg/ml, BD Biosciences, Franklin Lakes, NJ, USA) and propidium iodide (10 µg/ml; BD Biosciences) in the dark for 15 min at room temperature. Cell apoptosis was examined using flow cytometry (FACScan; BD Biosciences), according to the manufacturer's protocol.

Caspase activity. MUM-2B cells were seeded in a 6-well plate at 1x10⁶ cells/well for 24 h, then treated with 2.5, 5 and 10 µM damnacanthal for 24 h. Cells were washed twice with PBS and lysed in radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology) for 1 h at 37°C. Cells lysates were centrifuged at 12,000 x g for 15 min at 4°C, prior to determination of the protein concentration using a bicinchoninic acid (BCA) assay (Beyotime Institute of Biotechnology), according to the manufacturer's protocol. A total of 10 ng protein lysate was analyzed using the caspase-3 (acetyl-Asp-Glu-Val-Asp-p-nitroanilide) and caspase-9 (acetyl-Leu-Glu-His-Asp-p-nitroanilide) activity assay kits (both Beyotime Institute of Biotechnology), according to

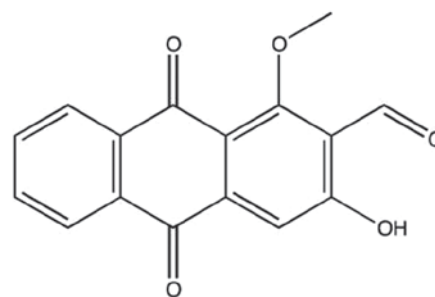


Figure 1. Structure of damnacanthal.

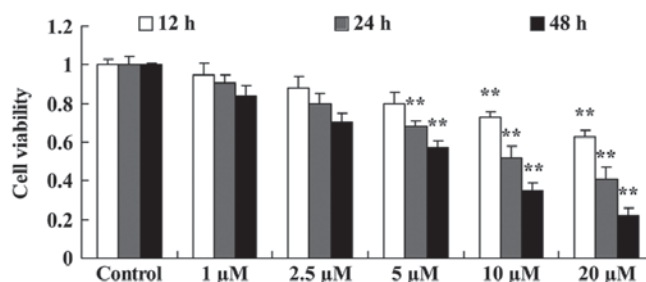


Figure 2. Damnacanthal inhibits of melanoma cell proliferation. MUM-2B cells were treated with 0, 2.5, 5, 10 or 20 µM damnacanthal for 12, 24 or 48 h. **P<0.01 vs. control group.

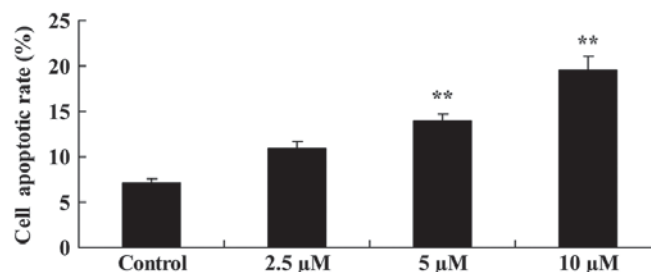


Figure 3. Damnacanthal induces apoptosis of melanoma cells. Flow cytometry was used to demonstrate the effect of increasing concentrations of damnacanthal on the apoptotic rate. **P<0.01 vs. control group.

the manufacturer's protocols. The optical density (OD) was measured using a microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 405 nm.

Western blot analysis. MUM-2B cells were seeded in a 6-well plate at 1x10⁶ cells/well for 24 h and were treated with 2.5, 5 and 10 µM damnacanthal for 24 h. Cells were washed twice with PBS and lysed using radioimmunoprecipitation assay buffer. Cells extracts were centrifuged at 12,000 x g for 15 min at 4°C, and the protein concentration was determined using a BCA assay. Protein lysates (50 µg) were separated by SDS-PAGE (8-10% gel) and transferred onto a nitrocellulose membrane (GE Healthcare, Chicago, IL, USA). The membrane was blocked with 5% skimmed milk in 0.1% TBS-Tween-20 prior to incubation with primary antibodies against the following: B-cell lymphoma 2 (Bcl-2)-associated X protein (Bax; cat. no. 5023; dilution, 1:2,000), p53 (cat. no. 2527; dilution, 1:2,000), p21 (cat. no. 2947; dilution, 1:2,000), NF-κB (cat. no. 8242; dilution, 1:2,000), cyclin D (cat. no. 2978;

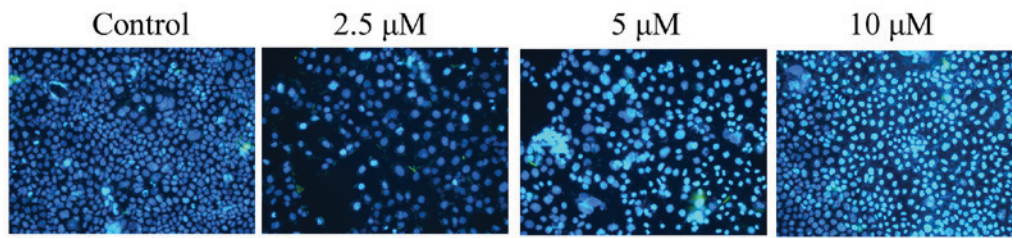


Figure 4. Damnacanthal induces apoptosis of melanoma cells. A DAPI assay (apoptosis was indicated by bright blue cells, x50 magnification) was used to illustrate the increase in apoptotic rate following damnacanthal treatment.

dilution, 1:2,000) and cyclin E (cat. no. 20808; dilution, 1:2,000) (all Cell Signaling Technology, Inc., Danvers, MA, USA), and GAPDH (dilution, 1:5,000; Bioworld Technology, Inc., St. Louis Park, MN, USA) at 4°C overnight. Following washing three times with TBS-0.1% Tween-20, membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (cat. no. 7074; dilution, 1:5,000; Cell Signaling Technology, Inc.) for 1 h at room temperature. Protein bands were detected using a chemiluminescence system (Beyotime Institute of Biotechnology) and quantified using ImageLab software (version 3.0; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. All results are expressed as the mean \pm standard deviation. Differences between groups were identified by one-way analysis of variance and Tukey's post-hoc test using SPSS software (version 17.0; SPSS, Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Damnacanthal inhibits proliferation of melanoma cells. The effect of damnacanthal on proliferation of melanoma cells was investigated using an MTT assay. Damnacanthal treatment decreased the proliferation of MUM-2B cells time- and dose-dependently. A treatment of 5–20 μ M damnacanthal significantly decreased cell proliferation after 24 and 48 h compared with untreated cells. Treatments of 10 and 20 μ M damnacanthal were able to significantly decrease the proliferation of MUM-2B cells after 12 h compared with untreated cells (Fig. 2).

Damnacanthal induces melanoma cell apoptosis. Flow cytometric analysis demonstrated that damnacanthal treatment induced melanoma cell apoptosis. Following treatment with 5 and 10 μ M damnacanthal for 24 h, apoptotic rate of MUM-2B cells was significantly increased compared with the untreated control cells (Fig. 3). A DAPI assay was used to stain apoptotic melanoma cells treated with damnacanthal for 24 h. As presented in Fig. 4, 5 and 10 μ M damnacanthal treatment observably increased the number of apoptotic cells after 24 h, compared with untreated control cells.

Damnacanthal induces caspase-3/9 activity in melanoma cells. To explore the underlying molecular mechanism of the effect of damnacanthal on apoptosis of MUM-2B cells, caspase-3/9 activity was analyzed using caspase activity assay

kits. As presented in Fig. 5, caspase-3/9 activity was significantly increased by 5 and 10 μ M damnacanthal treatment for 24 h compared with untreated control cells.

Damnacanthal treatment upregulates Bax, p53 and p21 protein expression levels in melanoma cells. Bax protein expression was analyzed by western blotting after 24 h treatment with damnacanthal. The assay revealed that 5 and 10 μ M damnacanthal significantly upregulated the Bax protein expression level in MUM-2B cells, compared with the untreated control cells (Fig. 6A, B). It was also investigated whether p53/p21 signaling may function in the anticancer effects of damnacanthal on melanoma cells. As presented in Fig. 6A, 6C–6D, p53 and p21 protein expression levels in MUM-2B cells were significantly increased following treatment with 5 and 10 μ M damnacanthal, compared with untreated control cells.

Damnacanthal treatment inhibits the protein expression level of NF- κ B, cyclin D and cyclin E in melanoma cells. Western blotting was used to determine the effect of damnacanthal on NF- κ B, cyclin D and cyclin E protein expression levels in melanoma cells. A significant inhibition of NF- κ B, cyclin D and cyclin E protein expression was observed in MUM-2B cells after 24 h treatment with 5 and 10 μ M damnacanthal compared with untreated control cells (Fig. 7).

Discussion

Melanoma is a highly malignant tumor originating in melanocytes and mainly identified in the skin; it is often caused by heritable genetic variation or environmental factors (3). The exogenous factor of greatest risk is exposure to ultraviolet irradiation. Malignant melanoma may also be derived from nerve sheath cells, which are able to generate melanin due to a mutation in nerve sheath cells, and the abnormalities of pigment generation and tyrosine metabolism (11). In the present study, damnacanthal treatment significantly decreased proliferation and increased the apoptotic rate of MUM-2B cells. Sukamporn *et al* (12) suggested that damnacanthal exhibits anticancer activity via downregulation of cyclin D1 expression.

p53 is a transcription factor which monitors the integrity of genomic cell DNA (13). p53 activates the transcription of the p21^{WAF1} gene, causing cell cycle arrest in G₁/S phase, whereas activation of the 14-3-3 gene leads to cell cycle arrest in the G₂/M phase, thus inhibiting tumor cell viability through cell cycle regulation (13). It also activates the expression of downstream genes, including Bax and NADPH oxidase activator, launching the apoptotic program and inhibiting the generation

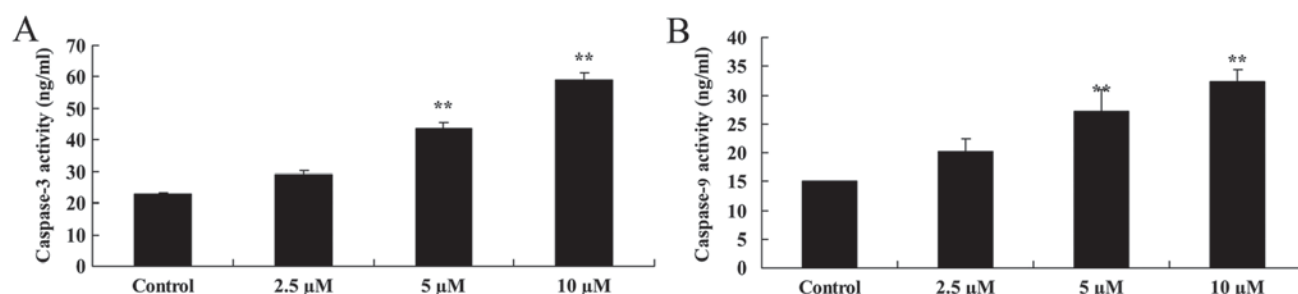


Figure 5. Damnacanthol induces caspase-3/9 activity in melanoma cells. (A) Damnacanthol treatment at 5 and 10 μ M increased caspase-3 activity in MUM-2B cells. (B) Damnacanthol treatment at 5 and 10 μ M increased caspase-9 activity in MUM-2B cells. **P<0.01 vs. control group.

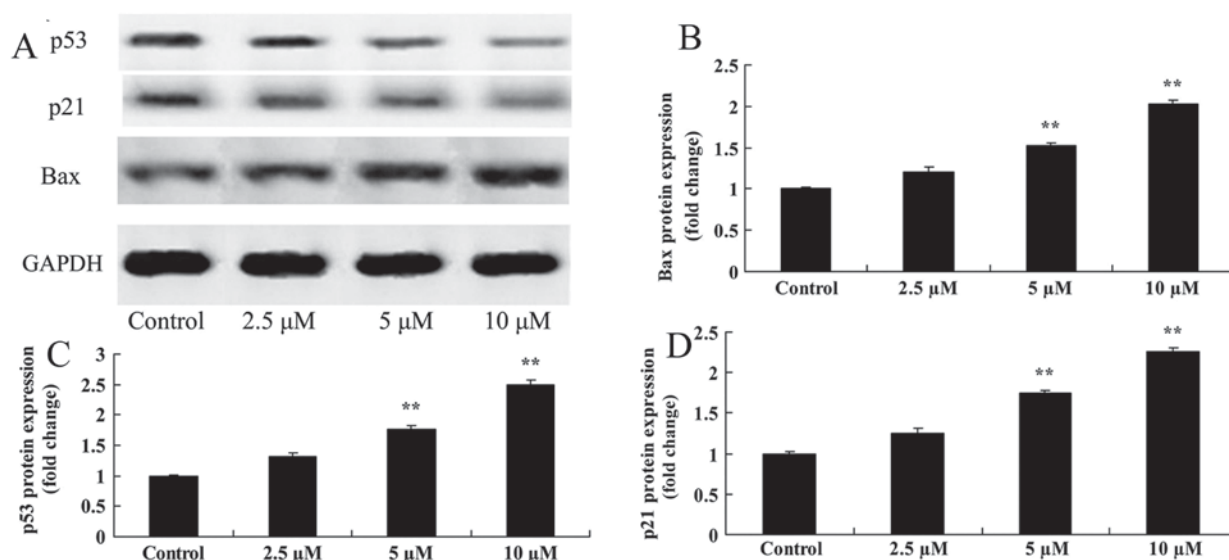


Figure 6. Damnacanthol upregulates Bax, p53 and p21 protein expression in melanoma cells. (A) Western blotting was used to demonstrate a dose-dependent increase in Bax, p53 and p21 protein expression level following damnacanthol treatment. Quantification and statistical analysis of (B) Bax, (C) p53 and (D) p21 protein expression in MUM-2B cells. Bax, B-cell lymphoma 2-associated X protein, p53, tumor protein p53. **P<0.01 vs. control group.

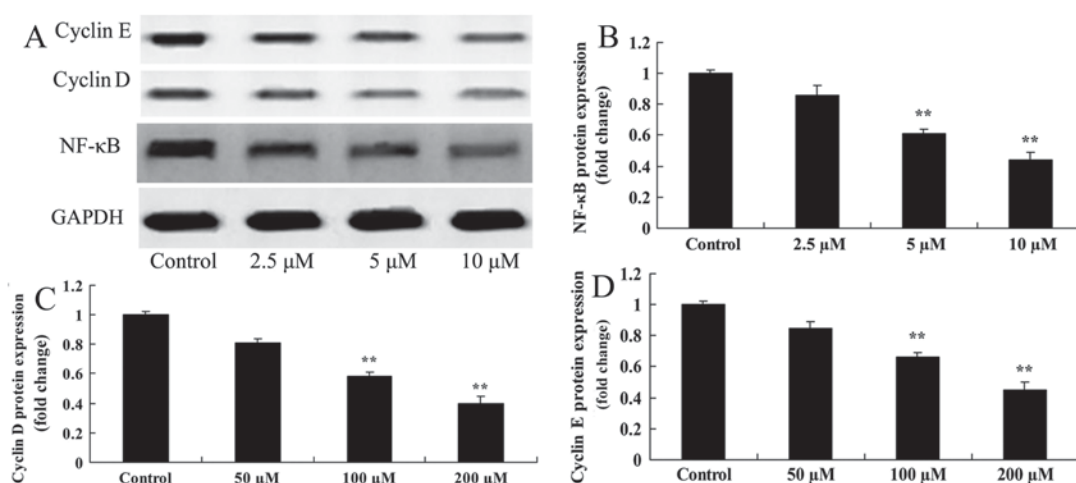


Figure 7. Damnacanthol inhibits NF-κB, cyclin D and cyclin E protein expression level in melanoma cells. (A) Western blotting was used to demonstrate a decrease in NF-κB, cyclin D and cyclin E protein expression level following damnacanthol treatment. Quantification and statistical analysis of (B) NF-κB, (C) cyclin D and (D) cyclin E protein expression. NF-κB, nuclear factor-κB. **P<0.01 vs. control group.

of cells with cancerous tendencies, thereby preventing malignant cell proliferation (14). Therefore, p53 serves an important

function in the maintenance of normal cell viability and function. However, wild-type p53 has poor stability, a short

intracellular half-life, often <20 min, and cannot be reliably identified using immunohistochemistry (15). The intracellular level and activity of p53 are finely regulated at the transcriptional, translational and post-translational levels, and by subcellular localization, among other processes (7). In the present study, it was demonstrated that damnacanthal significantly suppressed cyclin D and cyclin E protein expression, promoted caspase-3/9 activity, and induced Bax, p53 and p21 protein expression in melanoma cells. Aziz *et al* (16) reported that damnacanthal induced apoptosis by stimulating p53 and p21 expression in the breast cancer cell line MCF-7 (16).

NF- κ B is a transcription factor widely distributed in eukaryotic cells, which serves a key function in tumor cell proliferation, apoptosis, invasion, metastasis and angiogenesis (17). In recent years, it has been reported that tumor resistance to chemotherapeutic drugs is associated with NF- κ B (18). NF- κ B is able to be activated by a variety of factors, including pro-inflammatory cytokines, growth factors and cell stress; it may also be activated by chemotherapeutic drugs, including daunorubicin, doxorubicin and cisplatin and other chemotherapy drugs (19). NF- κ B is also a key regulator of apoptosis, and may induce the expression of anti-apoptotic factors, including survivin and Bcl-2 (20). NF- κ B activation is strictly regulated by inhibitor of NF- κ B, therefore conventional chemotherapy drugs are often accompanied by NF- κ B inhibitors, to inhibit the NF- κ B signaling pathway, decrease local recurrence and improve patient survival rate (21). The results of the present study demonstrated that damnacanthal significantly suppressed NF- κ B protein expression in MUM-2B cells. Kim *et al* (22) suggested that damnacanthal inhibits the NF- κ B signaling pathway in mast cells (22).

In conclusion, the results of the present study demonstrated that damnacanthal treatment inhibits cell proliferation, induces cell apoptosis, increases caspase-3/8/9 activity, upregulates the protein expression level of Bax, and downregulates the protein expression levels of cyclin D and cyclin E in melanoma cells through the p53/p21 and NF- κ B/cyclin/caspase-3 signaling pathways. These results suggest that damnacanthal may serve as a potential novel drug in patients with melanoma.

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Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

CL designed the experiment; XZ, PF, ZZ, XD, FX, YW performed the experiment; CL and XZ analyzed the data; CL wrote the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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