Original Research Article

Excisanin A suppresses proliferation by inhibiting hypoxia-inducible factor-1α expression in human hepatocellular carcinoma cells

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Abstract

Purpose: To investigate the effect of excisanin A on human hepatocellular carcinoma cells as well as to elucidate its mechanism of action.
Methods: Molecular docking was used to determine the binding characteristics of excisanin A to HIF-1α protein. The transcriptional activation and viability of excisanin A were assessed using Luciferase reporter and MTT assay. The HIF-1α protein in the nucleus was assayed using western blot and immunofluorescence. HIF-1α and VEGF mRNA levels were evaluated using reverse-transcription polymerase chain reaction (RT-PCR). Cell proliferation was determined by flow cytometry, as well as by EdU and clonogenic assays. In vivo tumor growth was assessed in a murine xenograft model of SK-Hep1 cells.
Results: Excisanin A inhibited HIF-1α transcriptional activation, as well as HIF-1α protein synthesis ($p < 0.001$). Excisanin A also reduced VEGF protein and mRNA expressions ($p < 0.001$). In addition, the compound inhibited the proliferation of hepatocellular carcinoma cells. and tumor growth in the xenograft tumor model.
Conclusion: Excisanin A is a potent HIF-1α inhibitor, supporting its potential development for human hepatoma therapy.

Keywords: Excisanin A, HIF-1α, Protein synthesis, Hepatoma therapy

INTRODUCTION

When oxygen levels are very low, HIF-1α transfers to the nucleus. Then, HIF-1α and HIF-1β begin the transcriptional program [4]. HIF-1α levels are closely related to VEGF expression involved in tumor angiogenesis and proliferation index [5]. As HIF-1α could promote tumor angiogenesis and growth, targeting HIF-1α may be a critical drug for cancer treatment. Many

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types of *Isodon* (Labiatae), a species of plant widely distributed throughout China, exhibit anti-inflammatory and anti-bacterial activities. *Isodon* diterpenoids have been identified to possess intense antitumor activity and very low toxicity, thus receiving considerable attention from both phytochemical and biological fields [6]. In this research, we discovered that excisanin A inhibited HIF-1α protein. Moreover, excisanin A suppressed the HIF-1α downstream genes such as VEGF, which is very important for tumor growth. Excisanin A also inhibited tumor cell proliferation. Based on these results, we further demonstrated that *in vivio*, excisanin A showed significant antitumor activity, which elicited no apparent toxicity in experimental animals.

EXPERIMENTAL

Cell lines and chemicals

Hep3B and SK-Hep1 cells (ATCC, USA) were routinely cultured in DMEM including 10% FBS and 1% penicillin/streptomycin. The hypoxic was kept in atmosphere at 1% O_2, 5 % CO_2 and 37°C. Dimethyl sulfoxide, cycloheximide and MG132 were purchased from Sigma-Aldrich. Excisanin A (≥ 98%) was extracted from *Isodon Macrocalyxin D,* and the structure of excisanin A is displayed in Figure 1 A.

Molecular docking

Molecular docking study was performed to detect the binding mode between excisanin A to HIF-1α protein using Autodock vina 1.1.2. The search grid of the HIF-1α was identified as center_x: -113.125, center_y: -55.376, and center_z: 12.189 with dimensions size_x: 15, size_y: 15, and size_z: 15.

Luciferase reporter assay

pGL3-HRE-Luciferase plasmid and pRL-CMV were co-transfected into SK-Hep1 cells. Then, cells were incubated with Excisanin A and under hypoxia for 12 h. Determined luciferase activity with a luciferase assay kit (Promega, USA).

MTT assay

After cells adhered, they were treated with excisanin A for 24 h. Then, living cells changed MTT (Sigma-Aldrich) to formazan, which dissolved in DMSO, producing blue-purple color.

Western blotting

Cell lysates were prepared as described previously [5]. The primary antibodies were HIF-

1α (Novus Biologicals, USA), phospho-p70S6K, Cyclin D1, phospho-ERK1/2, phospho-Akt, phospho-mTOR, phospho-eIF4E, phospho-SAPK/JNK (Cell Signaling Technology), Topo-I, VEGF (Santa Cruz, USA), α-tubulin (Sigma-Aldrich, USA) and c-Myc (Abmart, Shanghai, China). Finally, the appropriate secondary antibody was detected by enhanced chemiluminescence.

Immunofluorescence assay

Immunofluorescence assay was performed as described previously. Briefly, the cell nuclei were labeled with DAPI. The HIF-1α proteins appeared green and the nuclei appeared blue under confocal microscopy (Nikon, Japan) [6].

Reverse transcription-PCR (RT-PCR) analysis

SK-Hep1 cells treated with Exisanin A and isolated total RNA according to the manufacturer's instructions (Invitrogen, USA). GAPDH served as a housekeeping gene control. The bands were visualized by 3% agarose gel under UV light and photographed.

Flow cytometry analysis

Cell cycle was detected by propidium iodide staining, as described previously [8].

Clonogenic assay

Cells treated with excisanin A. Two weeks later, Colonies were fixed with 10 % formaldehyde and tinted with 1% crystal violet. The image was photographed with a camera.

EdU assay

Cells were plated in 96-well culture plates. Twenty-four hours later, cells were treated with excisanin A. Cells were incubated with EdU. Then, cells treated 1 × Apollo® reaction cocktail and the cell nucleus was tinted with Hoechst 33342.

Tumor xenografts

All mouse protocols were approved by the Yanbian University Institutional Animal Care and Use Committee. 1×10^7 cells in PBS were injected into the subcutaneous. Five days later, BALB/C nude mice (n = 5/group, five-week, male, Vital River, China) were given excisanin A three times per week. The body weight and tumor was measured with a caliper for 40 days and it was calculated following equation: (length × (width)^2)/2.

Immunohistochemical analysis

The paraffin-embedded sections of the tumor tissue were prepared for H&E staining and immunohistostaining. Histopathological changes and positive stained area was photographed by a photo microscope [2,9].

Statistics

Data are expressed as mean ± SD, and were compared using one-way ANOVA and Tukey's multiple comparison tests with the aid of SPSS software. $P < 0.05$ was considered statistically significant.

RESULTS

Excisanin A was identified as a HIF-1 inhibitor

We performed HIF-1α reporter assays, and found excisanin A suppressed hypoxia-induced reporter gene expression in a concentration-dependent manner (Figure 1 B). Additionally, viability at concentrations 3-30 μM of excisanin A after 24 h of treatment showed no obvious changes in Hep3B and SK-Hep1 cells, suggesting that the effects of excisanin A are not the result of cytotoxicity (Figure 1 C). Subsequently, Molecular docking assays revealed that excisanin A is properly bound to HIF-1α (Figure 2 A and B).
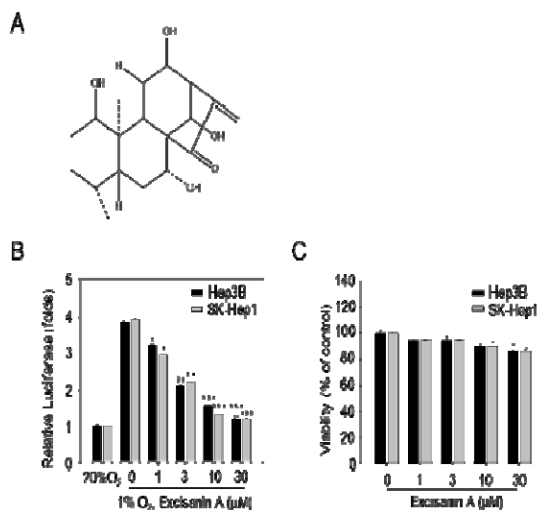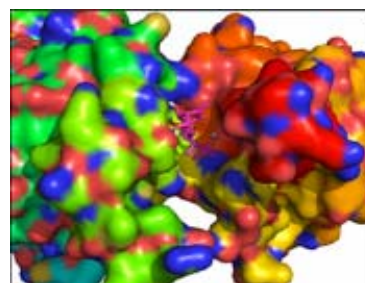
Figure 1: Excisanin A is a HIF-1 inhibitor. (A) Chemical structure of excisanin A. Chemical structure of excisanin A. (B) Excisanin A inhibited HIF-1α transcriptional activation. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ *vs* hypoxia group, n=3. (C) Cell viability was measured
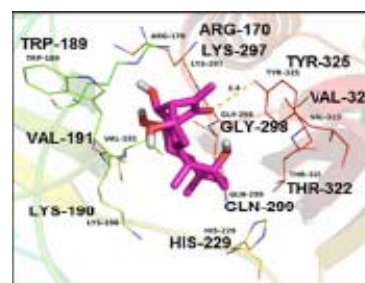
Figure 2: Computational modeling of Excisanin A binding to the HIF-1α

Excisanin A decreases HIF-1α protein levels

In Hep3B and SK-Hep1 cells, excisanin A significantly inhibited HIF-1α expression in a concentration-dependent manner in 1% O_2 (Figure 3 A). And, excisanin A inhibited the accumulation of HIF-1α at each time point in 1% O_2 (Figure 3 B). Next, immunofluorescence staining was performed. In Hep3B and SK-Hep1 cells, excisanin A (30 μM) nearly totally suppressed hypoxia-induced HIF-1α protein in the nucleus after 12 h of treatment (Figure 3 C).

Excisanin A inhibits HIF-1α protein synthesis

Excisanin A decreased HIF-1α protein levels in the presence of proteasome inhibitor (MG-132) (lanes 3 and 5 in Figure 4 A). The results indicated that excisanin A significantly inhibits HIF-1α protein synthesis. The use of the cycloheximide (CHX) inhibits protein synthesis. As displayed in Figure 4 B, while HIF-1α levels declined promptly in CHX treatment, excisanin A did not affect the HIF-1α degradation rate. Thus, excisanin A does not facilitate HIF-1α degradation. HIF-1α mRNA level was not altered with Excisanin A treatment in Hep3B and SK-Hep1 cells (Figure 5 A and 5 B). These findings suggested that excisanin A inhibited HIF-1α protein expression but did not inhibited HIF-1α mRNA expression.
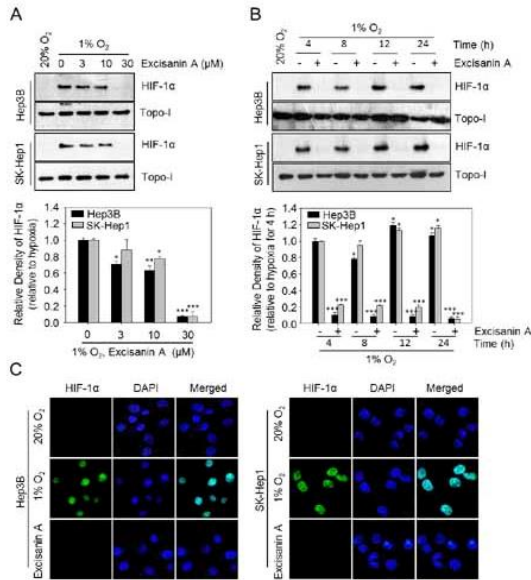
Figure 3: Excisanin A decreases HIF-1α protein levels. (A) Cells were incubated with excisanin A and incubated in hypoxia for 12 h. Western blot assay was performed. (B) HIF-1α expression was analyzed by immunoblotting. (C) HIF-1α distribution was detected by immunofluorescence in cells. The location and size of the nucleus were stained with DAPI. magnification = 400×

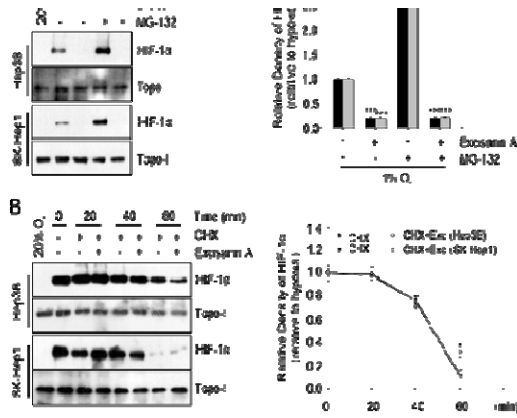**Figure 4** Excisanin A inhibits HIF-1α protein synthesis. (A) Cells treated with MG-132 (10 μM) for 30 min before adding excisanin A (30 μM). After 12 h, protein was analyzed by immunoblotting. *** $p < 0.001$ *vs* hypoxia group, n=3. (B) Cells were cultivated in 1 % O_2 atmosphere. After 4 h, CHX (10 μM) and excisanin A were added in culture media. After the addition of CHX for 15, 30, or 45 min, HIF-1α protein was analyzed by immunoblotting

Excisanin A decreases HIF-1α target genes expression

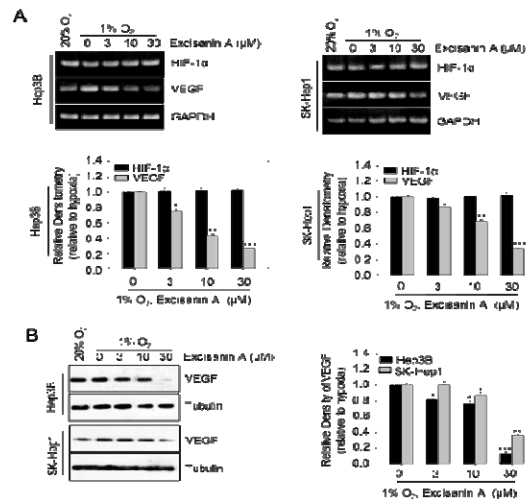Excisanin A inhibited VEGF mRNA and protein level in Hep3B and SK-Hep1 cells (Figure 5 A and B).

Figure 5: Excisanin A decreases expression of HIF-1α target genes. (A and B) The mRNA and protein were analyzed; *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$ *vs* hypoxia group, n = 3

Excisanin A inhibits cell cycle progression in the G1 phase

Hep3B cells were treated with excisanin A resulted in an increase of G1 phase cells from 56.28 to 61.14, 67.65, and 71.84 % in the concentrations of 5, 10, and 30 μM excisanin A. Similarly, treatment of SK-Hep1 with excisanin A also increases the number of G1-phase cells (Figure 6). In Figure 7, excisanin A inhibited cyclin D1 and c-Myc expression, thereby blocking cell cycle at the G1 phase.

Excisanin A inhibits the proliferation

EdU assay confirmed that excisanin A suppressed EdU-positive cells number, indicating that excisanin A suppressed Hep3B and SK-Hep1 cells proliferation *in vitro* (Figure 8 A). Clonogenicity of cells in excisanin A-treated groups were decreased with increasing concentration (Figure 8 B). Furthermore, MTT assay showed that excisanin A inhibits cell proliferation in both normoxia and hypoxic conditions (Figure 9 C and D).

Excisanin A inhibits tumor growth in a xenograft tumor model

As shown in Figure 10 A, excisanin A inhibits growth of hepatocellular carcinoma, while mice body weight was unchanged (Figure 10 B). After the last treatment, the tumor was harvested, and the representative tumor block was displayed in Figure 10 C. Consistent with above findings, excisanin A decreased HIF-1α protein in tumors (Figure 10 D).

Excisanin A inhibited the HIF-1α and VEGF expression in tumor (Figure 10 E). Taken together, our experiment confirmed that HIF-1α downregulation by excisanin A contributed to inhibit tumor growth and angiogenesis in tumor tissues.
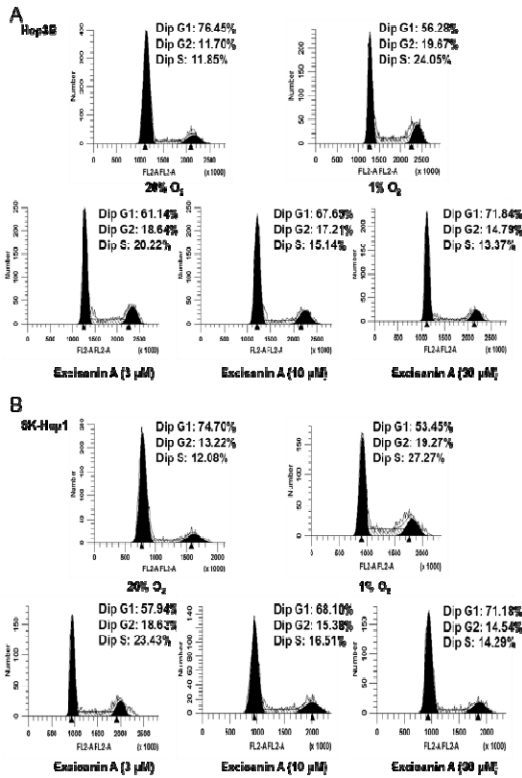

Figure 6: Excisanin A blocks cell cycle progression. Cells were cultured in 1% O_2 for 12 h and treated with different concentrations of excisanin A (A and B) Cycle progression was analyzed by flow cytometry. (B) Immunoblot showed protein expression. (significant at* *p* <0.05, ** *p* < 0.01, *** *p* < 0.001, n = 3)
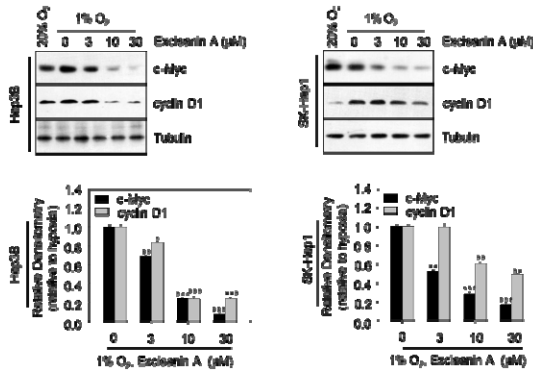

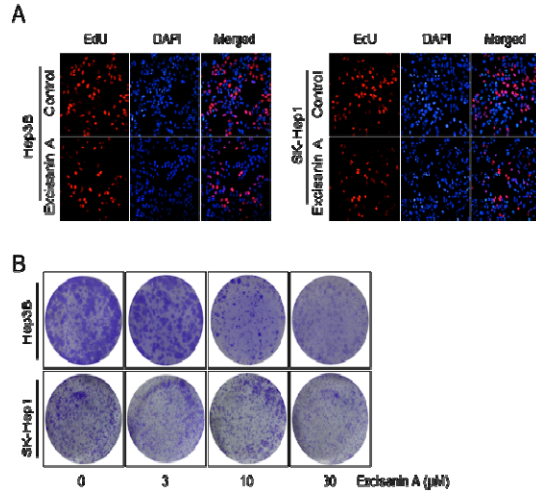**Figure 7:** Excisanin A blocks cyclin D1 and c-Myc expression

Figure 8: Excisanin A regulates the proliferation. (A) Treated cells with 30 μM excisannin A in normoxia for 12 h. Cells were observed by immunofluorescence staining. (B) The image was collected by the camera.
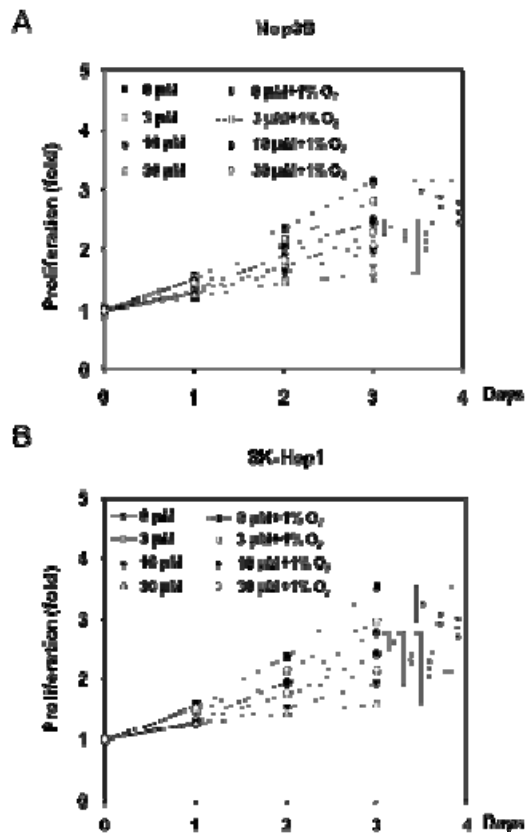

Figure 9: Excisanin A regulates the proliferation by MTT analysis; * *p* < 0.05, ** *p* < 0.01, *** *p* < 0.01 significant with respect to the normoxia control; # *p* < 0.05, ## *p* < 0.01, ### *p* < 0.01 significant with respect to the hypoxia control
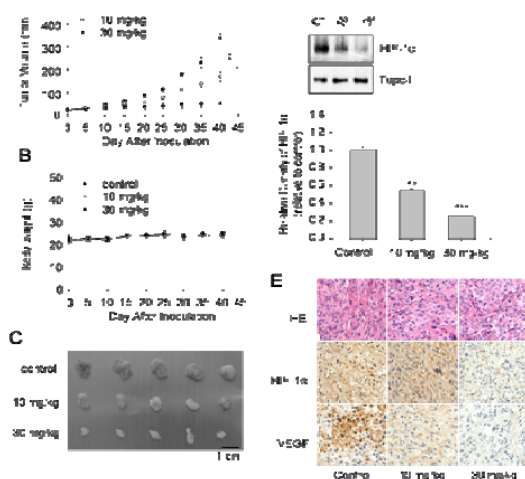
Figure 10: Excisanin A inhibits growth and tumorigenicity of hepatocellular carcinoma. (A) Tumor volume and (B) body weight was determined. (C) Photographs of mice bearing subcutaneously implanted tumor. (D) Immunoblots showed protein expression of HIF-1α. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ *vs* control group, n=5. (E) HE staining and immunohistostaining. Original magnification, 200×

Effect of excisanin A effects mTOR/p70S6K/4E-BP1 and MAPK pathways

Literatures have shown that PI3K-Akt-mTOR and MAPK mediate the translation of Hypoxia-inducible Factor 1α [10, 11]. Unfortunately, we found that excisanin A only inhibits phosphorylation of mTOR, whereas it promotes phosphorylation of Akt, p70S6K, eIF4E, JNK and ERK in Hep3B cells (Figure 11). It is a problem awaiting further study to determine the pathway by which excisanin A inhibits HIF-1α synthesis.
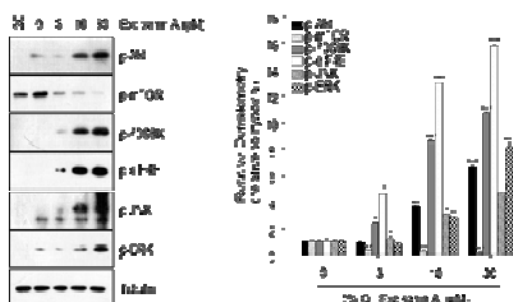

Figure 11: Excisanin A affects mTOR/p70S6K/4E-BP1 and MAPK pathways. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ *vs* hypoxia group, n = 3

DISCUSSION

Hypoxic conditions induce activation of the HIF-1α sub-unit, an important transcription factor for adaptation to hypoxic conditions within the tumor microenvironment. Thus, the development of HIF-1α inhibitors in cancer treatment is a huge challenge. In the process of trying to find anticancer agents from natural products, excisanin A, a natural ent-kaurane diterpenoid, was isolated from the traditional Chinese medicine *Isodon MacrocalyxinD*. Here, we found that excisanin A suppressed HIF-1α protein synthesis.

VEGF, a downstream target of HIF-1α, exerts multiple effects on tumor angiogenesis, including stimulating new blood and lymphatic vessel formation and increasing vascular permeability [12-16]. VEGF promotes the delivery of peripheral oxygen *via* stimulating angiogenesis, which involves the migration, proliferation and differentiation of the endothelial cell and the proteolysis of extracellular matrix. As expected, excisanin A decreased VEGF mRNA and protein levels.

We found that Excisanin A reduces cell proliferation by arresting the cell cycle and inhibits cyclin D1 and c-Myc protein level. Moreover, Figure 6C and 6D showed that the effects of excisanin A on cell proliferation are consistent in normoxia or hypoxic conditions. We examined HIF-1α and VEGF expression in the sections of tumor and found that excisanin A suppressed their expression in tumor tissues.

CONCLUSION

The findings of this study demonstrate that excisanin A suppresses HIF-1α protein synthesis in Hep3B and SK-Hep1 cells. Furthermore, excisanin A suppresses cancer cell proliferation by arresting cell cycle at the G1 phase. This mechanism may partly explain the anti-tumor mechanism of excisanin A, thus supporting its development as an anticancer drug.

DECLARATIONS

Acknowledgement

Conflict of interest

No conflict of interest to disclose with regard to this work.

Contribution of authors

We 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 the authors. Xuejun Jin and Changgao Jiang conceived and designed the study. Li Zhuo Han, Chunliu Mi and Ke Si Wang performed all the experiments and wrote. Hong Xiang Zuo and Zhe Wang prepared all the figures. Ming Yue Li, Zhi Hong Zhang, Xuejun Jin reviewed and edited the manuscript. Li Zhuo Han, Chunliu Mi and Ke Si Wang equally contributed to this work. All authors read and approved the manuscript.

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