

Pro apoptotic effect of neosedumoside III on human hepatocellular carcinoma hepG2 cells and SMMC 7721 cells through G0/G1 phase cell cycle arrest and regulate caspase-cascade pathway

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ABSTRACT

Purpose: NeosedumosideIII (Neo) is a megastigmanes and belongs to monocyclic sesquiterpenoids compound with antioxidant, anti-inflammatory and pharmacological activities. In order to explore the anti-cancer effect and possible mechanism of Neo, the study examined the anti-proliferation and apoptosis effect of Neo against human hepatocellular carcinoma HepG2 cells and SMMC-772 cells and related mechanism *in vitro*.

Methods: The anti-proliferation effect of Neo was detected on HepG2 cells and SMMC-772 cells by MTT assay and IC50 with increasing dose and

time. Cell cycle and apoptosis were detected by flow cytometer. The changes of Bcl-2, Bax, Caspase-3, Caspase-8 and Caspase-9 proteins were detected by western blotting.

Results: The results indicated that Neo could inhibited proliferation of HepG2 cells and SMMC-772 cells *in vitro* and promoted apoptosis, it significantly induced apoptosis of HepG2 cells and SMMC-772 cells arrested cell cycle at G0/G1 phase in a dose-dependent manner, reduce the expression of Bcl-2 protein, and increase the expression of Bax and Caspase-3, Caspase-8 and Caspase-9 proteins.

Conclusion: Neo could inhibit proliferation and induce apoptosis of HepG2 cells and SMMC-7721 cells *in vivo* which suggested that it might be served as a promising candidate for the treatment of liver cancer.

Keywords: Neosedumoside III; Hepatocellular carcinoma; HepG2 cells and SMMC-772 cells; Mechanism

INTRODUCTION

Liver cancer is one of the most common tumors in the world and mainly caused by chronic hepatitis, fatty liver and other liver diseases [1] it's the second leading cause of cancer-associated high morbidity and mortality all over the worldwide [2,3]. At present, the proportion of mortality and morbidity of liver cancer showed tendency to ascend and the relative survival rate was reduced that indicated a poor prognosis [4]. Liver cancer is not very sensitive to chemotherapy and radiotherapy, and the mainly used treatment methods are surgical resection. however, liver cancer due to its high invasive nature and most of the patients have developed to advanced stage at the time of diagnosis, lost the best time for treatment [5]. Therefore, the most important task is screening potential and effective compounds for the treatment of liver cancer.

Natural products isolated from Traditional Chinese Medicines are one of the important sources for anti-cancer drug which used as effective cancer treatment in China. NeosedumosideIII (Neo) is a megastigmanes and belongs to a class of monocyclic sesquiterpenoids with antioxidant, anti-inflammatory and other pharmacological activities [6,7]. A large number of literature reports monocyclic sesquiterpenoids are widely distributed in nature, and have inhibitory activity for human breast cancer cells MDA-MB-231, human colon cancer cells HCT116, Human liver cancer cells HepG2 and SMMC-7721, etc. [8-10]. Meanwhile, a megastigmanes has inhibitory activity for human lung adenocarcinoma cells A549, human prostate cancer cells PC3 and human breast cancer cells MCF7 [11]. However, Neo as a megastigmanes compound and the inhibition of Human liver cancer cells HepG2 and SMMC-7721 has not been reported, and the protective mechanism of cos against liver cancer is still unclear. Therefore, in order to explore the anti-cancer effect of Neo and its possible mechanism. The study evaluated the effect of Neo against liver cancer and the

mechanism of action of Neo on human liver cancer HepG2 cells and SMMC-772 cells *in vitro*.

In this study, the growth inhibit effect of Neo was detected on HepG2 cells and

SMMC-7721 cells by MTT assay. Cell cycle and apoptosis analysis were detected by

flow cytometry. The expression of Bax, Bcl-2 and Caspase-3, Caspase-8, and Caspase-9 proteins were analyzed by western blotting, the study were used to analyze whether Neo could be used as a promising candidate drug for the treatment of liver cancer. We found that Neo effectively inhibited cell proliferation and induced apoptosis in HepG2 cells and SMMC-7721 cells and its potential mechanisms underlying the effects, it indicate that Neo might be used as a potential drug for the liver cancer therapy.

MATERIALS AND METHODS

Reagents

Neo was bought from Herbpurify Company (Chendu, China, purity >98%) and dissolved in DMSO and stored at 4°C. Human Hepatocytes L-02 cells, Human Hepatocellular Carcinoma HepG2 cells and SMMC-7721 cells were purchased from Shanghai Institute of cell biology for Chinese Academy of Sciences (Shanghai, China). Dulbecco's minimum essential medium (DMEM, Gibco), fetal bovine serum (FBS, Gibco), streptomycin and penicillin (Invitrogen) were bought from Wolcavi Company (Beijing, China). Methyl Thiazolylte Trazdium assay kit (MTT), Dimethyl sulfoxide (DMSO), polyclonal antibodies (β -actin, Bax, Bcl-2, Caspase-3, Caspase-8, and Caspase-9) and secondary antibodies (goat-anti mouse), BCA protein kit, Trypsin, RIPA lysis buffer, cell cycle analysis kit and cell apoptosis detection kit were bought from Beyotime Company (Shanghai, China).

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Western Bright ECL prime Western-blotting detection reagent was bought from Ding-guo Company (Beijing, China).

Cell culture

Human Hepatocytes L-O2 cells, Human Hepatocellular Carcinoma HepG2 cells and SMMC-7721 cells were grown in DMEM (Gibco) containing 10% FBS (Gibco), and supplemented with streptomycin and penicillin (Invitrogen). Both types of cells were conducted at 37°C in a humidified 5% CO₂ incubator. Subsequently, the control group was cultured with DMSO whereas hepatocellular carcinoma HepG2 cells and SMMC-7721 cells were treated by 2, 4, 8, 16, and 32 µmol/L NEO for 0, 3, 6, 12, 24 and 48 h. For Neo treatment, the final concentration of DMSO added to the cells was <0.1%. In addition 0.1% DMSO group was used as a control.

Cell viability assay

MTT assay were used to assess viability and applied to measure L-O2 cells, HepG2 cells and SMMC-7721 cells viability. Three kinds of cells were plated 5 × 10⁴ cells/well in 96-well culture plates and treated with serial concentrations of Neo (2, 4, 8, 16, 32 µmol/L) for 48 h (various time periods) and MTT solution (5 mg/mL) was added to each well, DMSO was used as a control. Subsequently, the cell viability is measured by MTT assay; the IC₅₀ (The half maximal inhibitory concentration) values were calculated by linear-regression analysis and averages of three independent repeats being calculated.

Cell cycle analysis

After HepG2 cells and SMMC-7721 cells were treated with serial concentrations of Neo (2, 4, 8, 16, 32 µmol/L) and DMSO for 48 h, both types of cells were collected and centrifuged at 1000 g for 5 min to remove the culture medium, the cells were washed twice with PBS for cell cycle analysis. The cells were fixed with 70% ethanol at 4°C for 2 h, and the fixed solution was discarded, then cells resuspended 5 min by PBS and centrifuged at 1000 g for 5 min, PBS was discarded and stained with Propidium Iodide (PI), the cells were darkened at 4°C for 30 min, and cell cycle was detected by flow cytometry.

Cell apoptosis assay

After HepG2 cells and SMMC-7721 cells were treated with serial concentrations of Neo (2, 4, 8, 16, 32 µmol/L) and DMSO for 48 h and then cells were washed twice by PBS. Cells were resuspended with 500 µL binding buffer, Annexin V-FITC (5 µL) and PI solution (5 µL) were added and incubated at room temperature for 15 min in the dark, and the cell apoptosis were analyzed by flow cytometry (ACEA Novo-Cyte, Agilent Technologies Inc., USA). According to the instructions of apoptosis kit, the number of cells was calculated by cell analysis software (BD Inc., USA) for data analysis and the experiments were performed in triplicate.

Western blot assay

Human Hepatocellular Carcinoma HepG2 cells and SMMC-7721 cells were analyzed by western blotting. The total protein fractions were isolation and DMEM medium were removed. Subsequently, cells were washed twice by PBS and then lysed using cell by RIPA lysis buffer. The lysates were collected and centrifuged at 12000 g for 5 min at 4°C, protein concentrations were determined using the BCA protein assay. Samples were separated by 10% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and then transferred to PVDF membranes, and blots were blocked with 10% skim milk incubated with the primary antibodies Bax, Bcl-2, Caspase-3, Caspase-8, Caspase-9, anti β-actin overnight at 4°C. Then the membranes were incubated with secondary antibodies conjugated to HRP (Horse Radish Peroxidase), and gray scale analysis used by Image J software platform, the relative protein expression were expressed by the ratio of gray value of Bax, Bcl-2, Caspase-3, Caspase-8, and Caspase-9 protein to β-actin.

Statistical analysis

Data were expressed as the mean ± SD. Statistical comparisons of results were made using analysis of variance (ANOVA). Significant differences (*P<0.05, **P<0.01) whereas T-tests were used by SPSS software (version 19.0). P values with P<0.05 was the threshold for a statistical difference, whereas P<0.01 was the threshold for a significant difference. The graphs were generated with Origin version 9.0.

RESULTS

Effect of Neo on the proliferation of HepG2 cells and SMMC-7721 cells *in Vitro*

The chemical structure of Neo was shown in Figure 1A, which is a megastigmanes and belongs to monocyclic sesquiterpenoids compound. As shown in Figure 1B, the result of inhibitory effects showed that Neo effectively inhibited HepG2 cells proliferation in a dose-dependent and time-dependent (0, 3, 6, 12, 24, 36 and 48 h) compared with the DMSO group, and the IC₅₀ was 16.15 ± 1.27 µM. The findings were similar for SMMC-7721 cells (Figure 1C), which also showed that Neo effectively inhibited SMMC-7721 cells proliferation in a dose-dependent and time-dependent (0, 3, 6, 12, 24, 36 and 48 h) compared with the DMSO group, and the IC₅₀ was 15.87 ± 1.31 µM. In addition, NEO induced relatively lower cytotoxicity in L-O2 cells than in HepG2 and SMMC-7721 cells (Figure 1D) which shows it is safe for normal cells. In a word, other experiment and research were based on these results for further study *in vivo*.

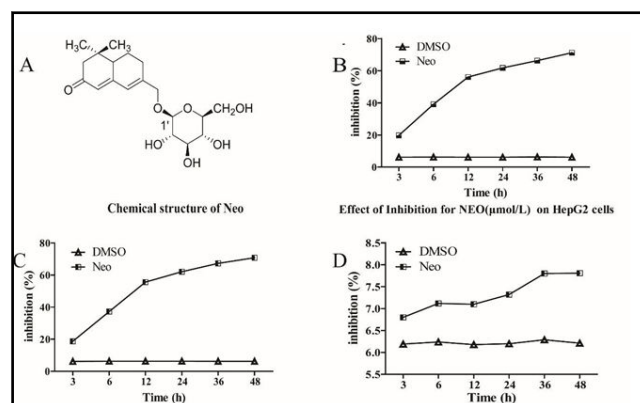


Figure 1: Effect of Inhibition for Neo (µmol/L) on HepG2 cells and SMMC-7721 cells *in Vitro*. A: The chemical structure of Neo. B: Effect of Inhibition for Neo on HepG2 cells. C: Effect of Inhibition for Neo on SMMC-7721 cells. D: Effect of Inhibition for Neo on L-O2 cells.

Effect of Neo on cell cycle distribution of HepG2 cells and SMMC-7721 cells *in Vitro*

The control group were cultured with DMSO whereas hepatocellular carcinoma HepG2 cells and SMMC-7721 cells were treated by 2, 4, 8, 16, and 32 µmol/L NEO for 48 h, the effect of Neo on the cell cycle of hepatocellular carcinoma HepG2 cells and SMMC-7721 cells were detected by PI staining and the results were shown in Figure 2. Compared with the control group, there was no significant change in cell percentage at G2/M stage in Neo group with different concentrations.

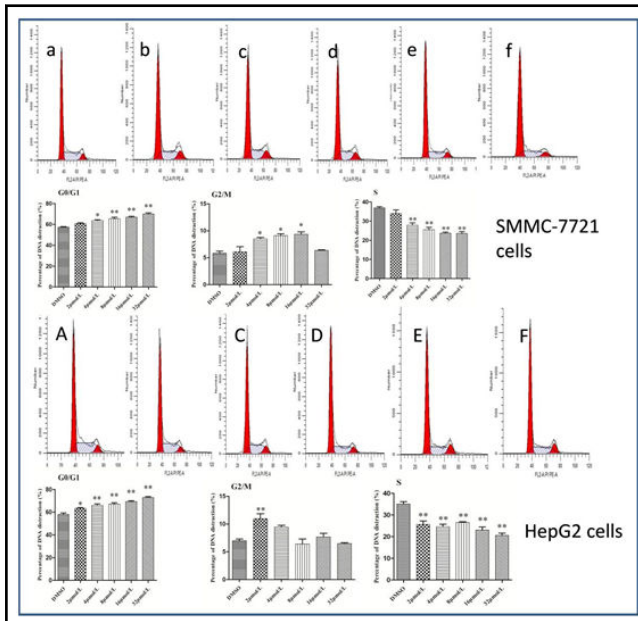


Figure 2: Effect of Neo on cell cycle distribution of HepG2 cells and SMMC-7721 cells *in Vitro*.

After all group cultured with either DMSO or for Neo 48 h, HepG2 cells and SMMC-7721 cells harvested fixed with ethanol and stained with PI, DNA content was determined by flow cytometry. The percentage of both cells at each phase of cell cycle was analyzed by Origin 9.0. A: DMSO, B: 2 $\mu\text{mol/L}$ cos, C: 4 $\mu\text{mol/L}$ cos, D: 8 $\mu\text{mol/L}$ cos, E: 16 $\mu\text{mol/L}$ cos, F: 32 $\mu\text{mol/L}$ cos. The lowercase letters have the same concentration as uppercase letters. Lowercase letters presents SMMC-7721 cells while uppercase letters present HepG2 cells. Data was expressed as mean \pm standard deviation from triplicate determinations. * $P < 0.05$, ** $P < 0.01$ vs. control.

While cell percentage at G0/G1 stage was significantly increased and cell percentage at S stage was significantly decreased. The change proportion of G0/G1 phase of HepG2 cells were caused by the different concentrations of Neo for (57.93 \pm 2.32) %, (63.34 \pm 1.22) %, (65.93 \pm 2.36) %, (67.02 \pm 2.03) %, (69.25 \pm 1.33) % and (72.92 \pm 1.43) %. And the change proportion of G0/G1 phase of SMMC-7721 cells were caused by the different concentrations of Neo for (57.26 \pm 1.03) %, (60.85 \pm 1.24) %, (63.60 \pm 1.60) %, (65.36 \pm 2.56) %, (66.90 \pm 1.48) % and (70.05 \pm 1.75) %. It was shown that Neo could arrest the cell cycle of hepatocellular carcinoma HepG2 cells and SMMC-7721 cells at G0/G1 phase and has a certain inhibitory effect on cell migration to G2/M stage.

Effect of different dose Neo on apoptosis in HepG2 cells and SMMC-7721 cells

To investigate the Neo induced apoptosis of hepatocellular carcinoma HepG2 cells and SMMC-7721 cells, the control group were cultured with DMSO whereas hepatocellular carcinoma HepG2 cells and SMMC-7721 cells were treated by 2, 4, 8, 16, and 32 $\mu\text{mol/L}$ NEO for 48 h, and the results were shown in Figure 3 and Figure 4. The apoptosis rate of HepG2 cells were caused by the different concentrations of Neo for (2.28 \pm 0.68) %, (6.86 \pm 1.30) %, (13.01 \pm 3.23) %, (20.08 \pm 3.10) %, (31.97 \pm 2.88) % and (50.67 \pm 5.89) %. And the apoptosis rate of SMMC-7721 cells was caused by the different concentrations of Neo for (1.82 \pm 0.30) %, (4.82 \pm 1.89) %, (11.44 \pm 2.54) %, (15.62 \pm 2.38) %, (30.55 \pm 2.92) % and (43.82 \pm 3.11) %. It was shown that the apoptosis rate of hepatocellular carcinoma HepG2 cells and SMMC-7721 cells increased with the increase of Neo concentration, which also indicated the potential anticancer mechanism of Neo.

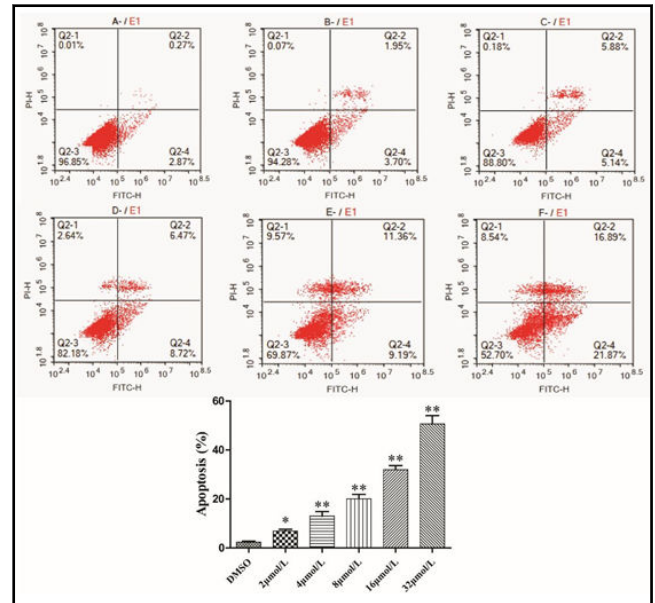


Figure 3: Effect of Neo on apoptosis in HepG2 cells *in Vitro*.

After all group cultured with either DMSO or for Neo 48 h, the HepG2 cells were stained with FITC-conjugated Annexin V in a buffer containing PI and analyzed by flow cytometry. The percentage of HepG2 cells categorized as apoptotic were analyzed by SPSS 19.0. A: DMSO, B: 2 $\mu\text{mol/L}$ cos, C: 4 $\mu\text{mol/L}$ cos, D: 8 $\mu\text{mol/L}$ cos, E: 16 $\mu\text{mol/L}$ cos, F: 32 $\mu\text{mol/L}$ cos. Data was expressed as mean \pm standard deviation from triplicate determinations. * $P < 0.05$, ** $P < 0.01$ vs. control.

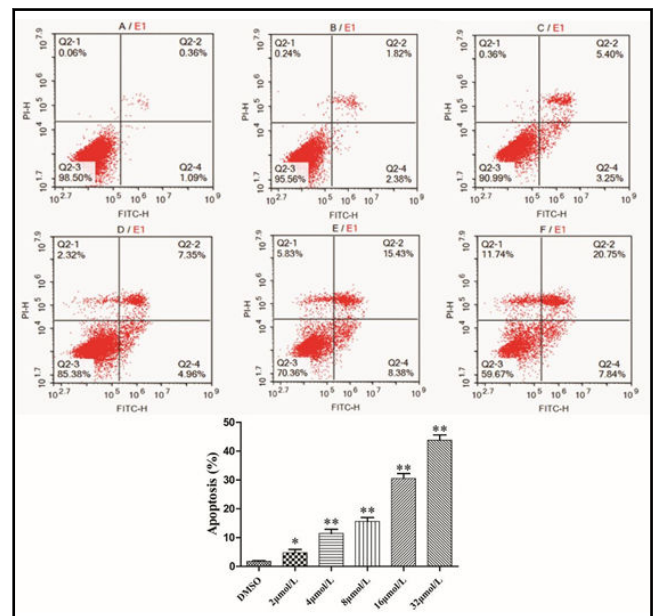


Figure 4: Effect of Neo on apoptosis in SMMC-7721 cells *in Vitro*

Effect of the Neo on the expression of Caspase-3, Caspase-8, and Caspase-9, Bax, and Bcl-2 in HepG2 cells and SMMC-7721 cells *in Vitro*.

As shown in Figure 5 and Figure 6. The expression of Bcl-2 family proteins and the caspase family proteins have the same change trend in both HepG2 cells and SMMC-7721 cells with the adding of Neo. It was found that Neo induced apoptosis of HepG2 cells and SMMC-7721 cells by up-regulating Bax, Caspase-3, Caspase-8 and Caspase-9 proteins and down-regulating Bcl-2 protein expression. All these changes were dependent upon the dose of Neo.

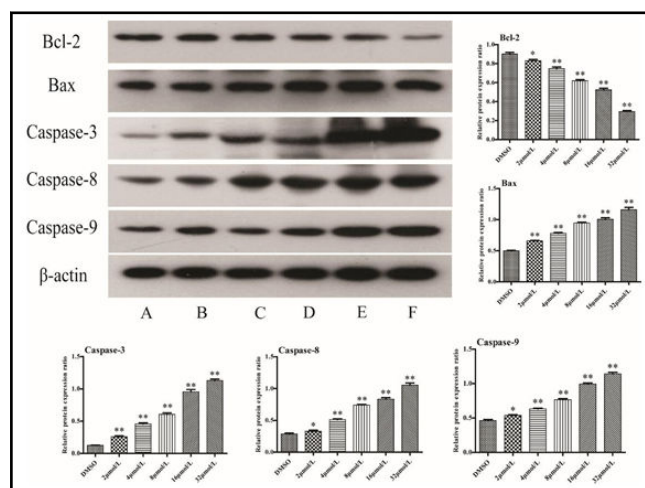


Figure 5: Effect of the Neo on the expression of Caspase-3, Caspase-8, and Caspase-9, Bax, and Bcl-2 in HepG2 cells *in Vitro*.

Western blotting analysis was used to detect protein expression levels of apoptotic markers on HepG2 cells, β -actin served as an internal control. A: DMSO, B: 2 μ mol/L cos, C: 4 μ mol/L cos, D: 8 μ mol/L cos, E: 16 μ mol/L cos, F: 32 μ mol/L cos. Data was expressed as mean \pm standard deviation from triplicate determinations. * P <0.05, *** P <0.001 vs. control.

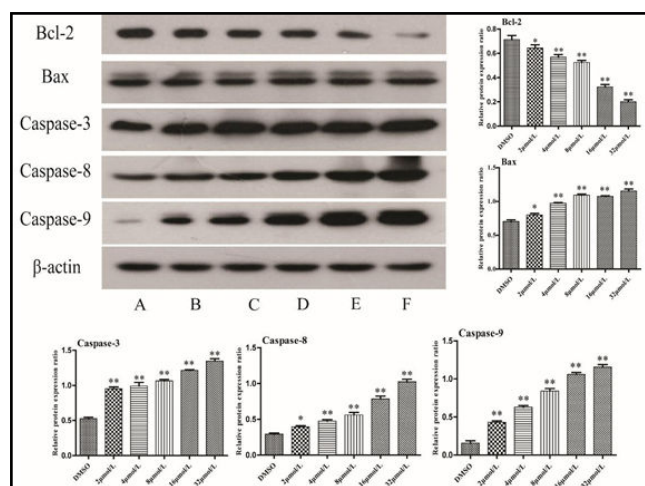


Figure 6: Effect of the Neo on the expression of Caspase-3, Caspase-8, and Caspase-9, Bax, and Bcl-2 in SMMC-7721 cells *in Vitro*.

Western blotting analysis was used to detect protein expression levels of apoptotic markers on SMMC-7721 cells, β -actin served as an internal control. A: DMSO, B: 2 μ mol/L cos, C: 4 μ mol/L cos, D: 8 μ mol/L cos, E: 16 μ mol/L cos, F: 32 μ mol/L cos. Data was expressed as mean \pm standard deviation from triplicate determinations.

DISCUSSION

The sesquiterpenes are widely distributed in nature. The monocyclic sesquiterpenoids are abundant in sesquiterpenes with many physiological activities including anti-inflammatory, antioxidant, anti-ulcer, cytotoxic activities and other physiological activities [12-14], among which anti-inflammatory and cytotoxic activities are the most prominent. There are many studies have shown that megastigmanes in different cancer cells have different cytotoxic activities [10] and Neo is a type of megastigmanes compound, in order to further verify the antitumor activity of Neo, the cytotoxic activities of Neo against HepG2 cells and SMMC-7721 cells *in vitro* was investigated. In this study, it was showed that Neo could inhibit the proliferation of HepG2 and SMMC-7721 cells and in a dose-dependent.

Carcinogenesis is a long and multistep process affected by many factors. The cell cycle is a primary factor which is a very sophisticated process and different compounds have different effects on the cell cycle, the G₀/G₁ and G₂/M phases are critical points in this process. It was found that the volatile oil significantly inhibited the proliferation of hepatocellular carcinoma HepG2 cells and SMMC-7721 cells cultured *in vitro* may be due to the increase of G₂/M phase and S phase, accompanied by the decrease of G₀/G₁ phase [15]. The effect of Rosiglitazone may be prevent the retention of hepatocellular carcinoma HepG2 cells in G₀/G₁ phase and inhibit the cell migration to G₂/M phase to a certain extent [16]. The Drug-containing serum of *Clerodendrum Bunge* blocks hepatocellular carcinoma MHCC97-H cells from G₂/M phase to G₀/G₁ phase and further reducing S phase, thereby interfering with DNA replication to inhibit cell proliferation [17]. Therefore, it is important to analyze the effect of substances on cell proliferation by detection for the cell cycle, our study found that the Neo could block the cell cycle arrest in G₀/G₁ phase, it indicated that Neo can block the cell cycle of both types of cells to some extent and it was a great significance for the study of anticancer mechanism.

Apoptosis is a physiological process of cells. In recent years, many studies have shown that the occurrence and development of tumors were related and may be caused by abnormalities in the process of cell apoptosis [18,19]. Bcl-2 proteins and Bax proteins belongs to Bcl-2 family and then were key factors in the process of cell apoptosis which were expressed abnormally in various tumors, and then have become one of the targets of anticancer drugs [20]. Cell apoptosis may be induced by inhibition of Bcl-2 or activation of Bax [21], the lower the ratio of Bcl-2/Bax and the higher the mortality of the cell, therefore, the up-regulation of Bax protein expression or the down-regulation of Bcl-2 protein expression might be related to the apoptosis of various tumor cells including liver cancer [22]. In addition, the study indicated that there are two apoptotic pathways including the "intrinsic" cytochrome C/Caspase-9 pathway and the "extrinsic" Caspase-8 pathway [23], caspase family proteins were also play a key role of cell apoptosis. Caspase-9 as a promoter of caspase which were necessary for apoptosis signal through mitochondrial pathways and activated on apoptotic complex, and thus activate caspase-3 [24]. Caspase-8 was also as a promoter of caspase and thus activate caspase-3 eventually leads to cell apoptosis [25]. When Caspase-3 was activated by upstream pathway and as a key "killer" of cell apoptosis [26] and induces cascade reactions leading to cells apoptosis [27]. Research suggests that costunolide can activate caspase-3 to mediate apoptosis of esophageal cancer cells by up-regulation of Bax protein expression and down-regulation of Bcl-2 protein expression [28], breast cancer MCF-7 cells apoptosis induced by curcuminol through regulating the expression of Bcl-2 protein and Bax protein and down-regulating the ratio of Bcl-2/Bax [29], and many other studies have shown that inducing apoptosis was important for inhibiting the development of liver cancer [30]. Therefore, the study investigated the effect of Neo on the expression levels of the key regulators of apoptotic pathways in HepG2 cells and SMMC-7721 cells including Bax, Bcl-2, Caspase-3, Caspase-8 and Caspase-9. In this study, western blotting analysis showed that Neo induced apoptosis of HepG2 cells and SMMC-7721 cells by up-regulating the expression of Bax, Caspase-3, Caspase-8 and Caspase-9 proteins, and down-regulating the expression of Bcl-2 protein. The results of the study were consistent with those in the literature, and it was indicating that Neo promoted apoptosis in liver cancer cells and has significance for the study of anti-cancer mechanisms.

CONCLUSION

In conclusion, Neo showed inhibition effect on proliferation of HepG2 cells and SMMC-7721 cells *in vitro*. The survival rate of the two cells decreased and cell apoptosis rate increased in a dose-dependent and time-dependent way. The mechanism of antitumor effect may be related to cell cycle arresting in the G₀/G₁ phase. The up-regulating expression of Bax, Caspase-3, Caspase-8 and Caspase-9 proteins and down-regulating the expression of Bcl-2 proteins in both cells may also show the mechanism of cell apoptosis when the dose of Neo is increasing. This study suggested that Neo may be a promising candidate or major compound for the

development of liver cancer, providing data support for further animal and clinical trials.

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DATA AVAILABILITY STATEMENTS

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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