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Original Research Article

Plumericin inhibits growth of liver carcinoma cells via downregulation of COX-2 and VEGF

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Abstract

Purpose: *To investigate the antitumor effect of plumericin on hepatocellular carcinoma and the underlying molecular mechanism(s).*
Methods: *Invasion of cancer cells was measured with matrigel Transwell assay, while COX 2 and VEGF mRNA expressions were determined using quantitative polymerase chain reaction (qPCR)*
Results: *Plumericin caused dose-dependent reductions in proliferations of Hep 3B and Hep G2 cancer cells. The degrees of proliferation of Hep 3B cells were 91, 84, 72, 57, 42 and 29 % at plumericin concentrations of 5, 10, 15, 20, 25 and 30 µM, respectively. In Hep G2 cells plumericin treatment at doses of 5, 10, 15, 20, 25 and 30 µM decreased proliferation to 89, 78, 64, 53, 42 and 30 %, respectively at 72 h. Treatment of Hep 3B cells at plumericin doses of 20, 25 and 30 µM led to induction of apoptosis in 41.23, 56.76 and 68.54 % of cells, respectively after 72 h. Plumericin suppressed the invasion potential of Hep 3B cells in a dose-dependent manner. Compared to control, the proportion of Hep 3B cells in G2/M phase of cell cycle increased significantly at doses of 20, 25 and 30 µM. Plumericin treatment of Hep 3B cells led to significant decrease in expressions of COX 2 and VEGF.*
Conclusion: *Plumericin suppresses liver cancer cell growth in vitro and in vivo by inhibition of COX 2 and VEGF expressions. Thus, it may be used for the treatment of liver cancer.*

Keywords: *Metastasis, Aflatoxins, Plumericin, Matrigel, Cyclooxygenase*

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INTRODUCTION

Hepatocellular carcinoma (HCC), also known as primary liver cancer is the third leading cause of death due to cancer throughout the world, and it affects 600.000 people every year [1,2]. In most of the cases, traditional chemotherapy has been found to be ineffective [3]. It has been reported that HCC is one of the most resistant types of the carcinomas, with respect to chemotherapy and radiotherapy [4]. It metastasizes easily to the lymph nodes, pulmonary tissues, bones, adrenal glands and skull [4].

Although surgical intervention has been found to increase the 5-year survival in some patients, the results are not satisfactory in majority of cases [5]. Thus, research on the discovery and development of new therapeutic strategy for the HCC is of immense importance. Hepatocellular

carcinoma (HCC) arises from ingestion of aflatoxins, nitrosoamines, alcohol addiction, hepatitis and chronic liver diseases [6]. Chronic hepatitis leading to cirrhosis is responsible for high incidence of HCC in China [7-9]. Natural products obtained from plant origin have been found to be very effective in the treatment of cancer [10]. Some natural products exhibit their effects through induction of apoptosis and cell cycle arrest, and also by synergizing with phosphatidylinositol 3-kinase [11,12].

Traditional Chinese Medicine in combination with modern chemotherapy has been reported to yield some satisfactory results in HCC treatment [13]. Traditional Chinese Medicine is preferred for carcinoma treatment because the active ingredients directly inhibit the growth and proliferation of cancer cells [14-17], and improve the immune system [18].

The present study investigated the effect of plumericin on proliferation and invasive potential of liver cancer cells *in vitro*, and its effect on the growth of liver tumor *in vivo*. The effect of plumericin on the expressions of COX-2 and VEGF was also investigated.

EXPERIMENTAL

Cell culture

The Hep 3B and Hep G2 liver cancer cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc.) medium containing 10 % fetal bovine serum (PBS, Gibco; Thermo Fisher Scientific, Inc.) at 37 ˚C in an atmosphere containing 5 % CO_2.

MTT assay

The Hep 3B and Hep G2 cells were seeded into 96-well plates pre-coated with agarose (0.6 %) at a density of 2 x 10^5 cells/well in RPMI-1640 medium. After overnight incubation, plumericin at doses of 5, 10, 15, 20, 25 and 30 µM was added to different wells of the plate, and the plates were incubated at 37 ˚C for 72 h. Following incubation, the medium was removed from the plates and the cells were incubated for 4 h with 20 µL MTT solution (5 mg/mL in PBS). Then, DMSO (150 µL) was added to each well to solubilize the resultant formazan crystals, and the absorbances of the solutions were read at 570-nm in an enzyme-labeling instrument (ELx800 Absorbance Microplate Reader type, BioTek Instruments, Inc., Winooski, VT, USA).

Apoptosis assay

Apoptosis induction in Hep 3B cells was determined with flow cytometry using Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) (BD) method. Annexin V/FITC kit (BD Biosciences, San Jose, CA, USA) was used in accordance with the instructions provided in the user manual. The Hep 3B cells were incubated for 72 h with 20, 25 and 30 µM plumericin, harvested, and exposed to HEPES binding buffer along with Annexin V-FITC and PI at room temperature for 20 min. The cells were then subjected to flow cytometric analysis in a FACSCanto™ flow cytometer (BD Biosciences).

Cell invasion assays

Invasion of Hep 3B cells through matrigel basement membrane (Sigma-Adrich, Carlsbad, CA, USA) was determined using the procedure reported earlier [19]. The Hep 3B cells (1 x 10^5) were plated onto matrigel-coated transwell inserts (BD Biosciences) and treated with plumericin at doses of 20, 25 and 30 µM for 72 h. Untreated cells served as control. The cells that invaded through matrigel-coated inserts were subjected to HEMA staining, counted and subsequently photographed under a light microscope.

Cell cycle analysis

Hep 3B cells were distributed into 6-well plates (2.5 x 10^5 cells/well) and incubated with plumericin at doses of 20, 25 and 30 µM for 72 h. Untreated cells were used as control. Then the cells were subjected to trypsinization, washed in phosphate-buffered saline (PBS), and subsequently fixed in 70 % ethanol. The fixed cells were stained with PI (0.5 mg/mL) in PBS and RNase A (50 mg/mL). Flow cytometric analysis was performed using flow cytometer. ModiFit LT™ software version 4.0 (BD Biosciences, Franklin Lakes, NJ, USA) for the data analysis.

Assay of COX-2 and VEGF expressions

Hep 3B cells were treated for 72 h with plumericin at doses of 20, 25 and 30 µM, with untreated cells as control. The cells were then collected for extraction of total RNA using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). The template for cDNA was constructed for performing qPCR with specific primers for cyclooxygenase-2, vascular endothelial growth factor and β -actin. Micro-capillary tube employing a Light Cycler™ (Roche Diagnostics, Basel, Switzerland) in combination with SYBR® Green Tag ReadyMix™ (Sigma-Aldrich) was

used for qPCR assays for transcriptions of cyclooxygenase-2 and vascular endothelial growth factor. The reaction mixture in the micro-capillary tube contained nuclease-free water (7.1 µL), SYBR reagent (10 µL), template cDNA (0.5 µL), magnesium chloride (1.6 µL; 25 mM), and primer mixture (0.8 µL; 25 pmol/µL). The conditions used for cycling were as follows: 30 sec denaturation at 95˚C, 5 sec annealing at 55 ˚C, 25 sec extension at 72 ˚C and 1 sec detection at 80 ˚C over 30 cycles. The products of PCR were subjected to electrophoresis using agarose gel (2.0 %) and photographed under UV.

Animals and treatment

A total of twenty BALB/c male mice (4-weeks-old) were purchased from the Beijing (HFK Bioscience Co., Ltd). The mice were kept in plastic cages under 12 h light/dark cycles, 25˚C temperature and 48 % humidity. The animals were provided food and water *ad libitum*. The mice, after one-week of acclimatization to laboratory atmosphere, were assigned to four groups of 5 mice each. Hep 3B cells (2.5 x 10^6) were injected subcutaneously into the mice belonging to untreated (control), 25 and 30 mg/kg treatment groups. At day 7 of injection, mice in the two treatment groups were given 25 or 30 mg/kg of plumericin daily up to one month of Hep 3B cell administration. The animals in the untreated and normal control groups received normal saline alone. After one month, the mice were sacrificed under isoflurane anaesthesia, and the tumor tissues were excised. The animal experimental protocols used in this study were approved by the Laboratory Animal Committee of the China Medical University (approval no. 20164351) and conducted according to the guidelines of "Principles of Laboratory Animal Care" (NIH publication no. 85-23, revised 1985).

Statistical analysis

Data are expressed as mean ± standard deviation (SD, n = 3). Statistical analysis of data was performed by one-way analysis of variance (ANOVA) with the SPSS software, version 11.5 (SPSS, Inc., Chicago, IL, USA). Differences were taken as significant statistically at $p < 0.05$.

RESULTS

Plumericin inhibited the proliferation of Hep 3B and Hep G2 liver cancer cells

Plumericin caused dose-dependent reduction in proliferation of Hep 3B and Hep G2 cancer cells. The degrees of proliferation were 91, 84, 72, 57, 42 and 29 % at plumericin doses of 5, 10, 15, 20, 25 and 30 µM, respectively, in Hep 3B cells (Figure 1). In Hep G2 cells, plumericin treatment at doses of 5, 10, 15, 20, 25 and 30 µM decreased proliferation to 89, 78, 64, 53, 42 and 30 %, respectively after 72 h (Figure 1).

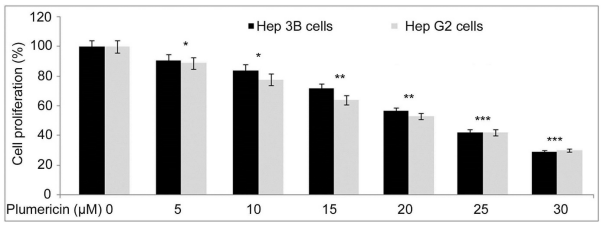

Figure 1: Effect of plumericin on the proliferation of Hep 3B and Hep G2 cancer cells. The cells, after 72 h of incubation with 5, 10, 15, 20, 25 and 30 µM plumericin, were subjected to MTT assay. Data are presented are mean ± standard deviation of triplicate experiments. *$p < 0.05$, **$p < 0.02$ and ***$p < 0.02$, compared to control cells

Plumericin induced apoptosis in Hep 3B cells

Treatment of Hep 3B cells with plumericin at doses of 20, 25 and 30 µM to induction of apoptosis in 41.23, 56.76 and 68.54 % of cells, respectively after 72 h (Figure 2). The percentage of apoptotic cells in untreated control cultures of Hep 3B cells was 1.76 % (Figure 2).

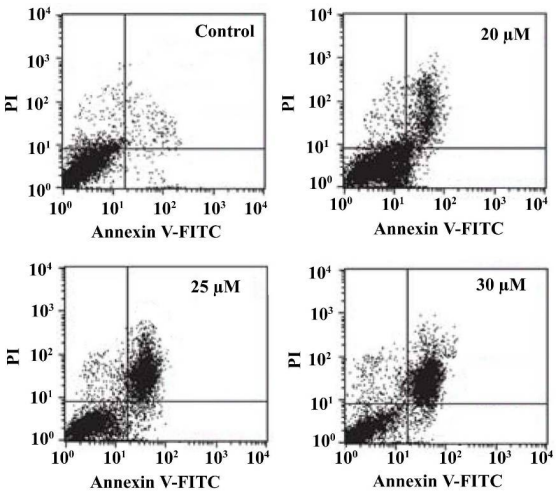

Figure 2: Effect of plumericin on apoptosis in Hep 3B cells. The cell cultures were incubated with plumericin at doses of 20, 25 and 30 µM for 72 h, and then subjected to flow cytometry using Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI)

Plumericin inhibited invasion of Hep 3B cells

Hep 3B cells were incubated with plumericin at doses of 20, 25 and 30 µM, and their invasion potential were determined using the Transwell chamber assay. It was found that plumericin treatment led to suppression of invasion potential

of Hep 3B cells in a dose-dependent manner (Figure 3). The suppression of Hep 3B cell invasion potential was significant at all the three tested doses of plumericin (Figure 3).

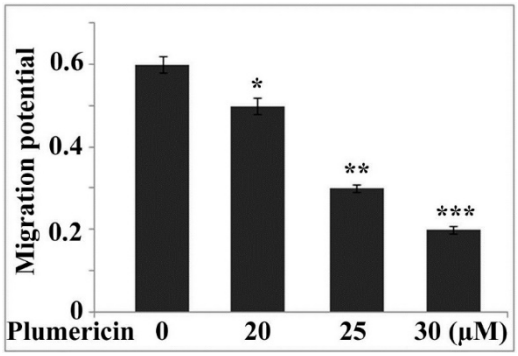

Figure 3: Effect of plumericin treatment of Hep 3B cells. The cells, after incubation with 20, 25 and 30 µM doses of plumericin, were subjected to Transwell invasion assay. Quantitative analysis of Transwell invasion assay showed that plumericin treatment significantly suppressed liver cancer cell invasion; *$p < 0.05$, **$p < 0.02$ and ***$p < 0.02$ vs. control cells. Images captured at a magnification of x100

Plumericin caused G2/M phase cell cycle arrest in Hep 3B cells

The effect of plumericin on cell cycle in Hep 3B cells was studied at doses of 20, 25 and 30 µM. Compared to control, the proportion of Hep 3B cells in G2/M phase of cell cycle was increased significantly at 20, 25 and 30 µM (Figure 4). However, the proportion of cells in S phase of cell cycle was subsequently decreased. In G2/M phase, the percentages of Hep 3B cells were 42.21, 54.89 and 59.75 on treatment with 20, 25 and 30 µM plumericin, respectively, for 72 h, while the percentages of cells in S phase were 16.56, 12.36 and 8.71, respectively on treatment with 20, 25 and 30 µM doses of plumericin. In G1 phase, there were 38.86, 31.12 and 30.63 % of cells due to treatment with plumericin at doses of 20, 25 and 30 µM, respectively. These results are shown in Figure 4.

Plumericin treatment led to inhibition of COX-2 and VEGF expressions in Hep 3B cells

After 72 h of incubation with 5, 10, 15, 20, 25 and 30 µM plumericin, the Hep 3B cells were examined for expressions of COX-2 and VEGF mRNAs using RT-PCR analysis. The results showed that plumericin led to significant and concentration-dependent decreases in the expressions of COX-2 and VEGF mRNAs, relative to the control group (Figure 5). The down-regulations of COX-2 and VEGF expressions were significant as from 10 µM plumericin at 72 h (Figure 5).

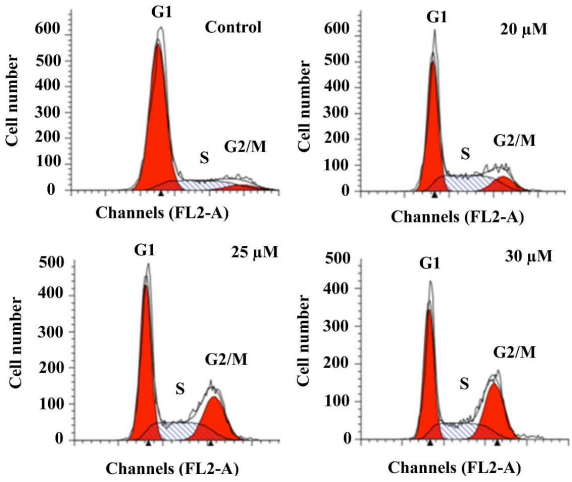

Figure 4: Effect of plumericin on cell cycle arrest in Hep 3B cells. Following incubation with 20, 25 and 30 µM plumericin for 72 h, the cells were washed, fixed, stained with propidium iodide, and examined for DNA content using flow cytometry

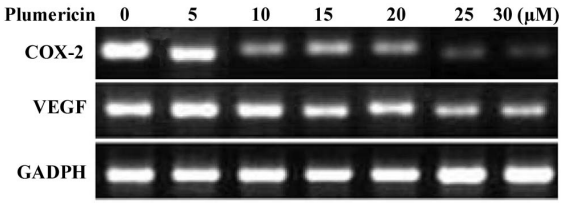

Figure 5: Plumericin reduced expressions of COX-2 and VEGF mRNA in Hep 3B cells. The cells were incubated for 72 h with plumericin and then subjected to RT-PCR analysis for expressions of COX-2 and VEGF mRNA

Plumericin inhibited liver cancer growth *in vivo*

Tumor weight was significantly lower in the plumericin-treated groups than in the control animals. However, tumor weight in animals treated with 30 mg/kg of plumericin was lower than that of 25 mg/kg treatment group. The tumor weights in control, untreated, 25 mg/kg plumericin treatment and 30 mg/kg plumericin treatment groups were 0, 2.8, 1.2 and 0.4 g, respectively.

DISCUSSION

The present study investigated the effect of plumericin on cell proliferation, invasion potential, expression of COX-2 and VEGF expression in liver cancer cells. Plumericin inhibited proliferation, induced apoptosis, suppressed invasion and downregulated the expressions of COX-2 and VEGF in liver cancer cells. Moreover,

plumericin inhibited tumor growth in mice model of liver cancer.

In tumor tissues, the rate of cell proliferation is exceptionally high, when compared to normal tissues. In the current study, plumericin caused reduction in the proliferation of Hep 3B and Hep G2 liver cancer cells. The inhibition of liver cancer growth by plumericin provided the basis for investigation of the mechanism involved in its action. Apoptosis is a very important and highly regulated biological process involved in the removal of unwanted cells from the body tissues. Abnormalities in the apoptotic process have been implicated in the etiology of cancer [20,21].

In the current study, flow cytometry showed that plumericin treatment markedly induced apoptosis in Hep 3B cells. Cell cycle constitutes a series of ordered steps each of which is regulated by checkpoints [22]. These checkpoints regulate cell cycle progression through extracellular signal detection, protein synthesis and DNA integrity [22]. Disorder in cell cycle is the leading cause of uncontrolled cell division. In the present study, flow cytometric analysis showed that plumericin treatment of Hep 3B cells arrested cell cycle in G2/M phase. This finding suggests that plumericin inhibits liver cancer cell growth by arresting cell cycle progression. In various types of tissues, the inducible enzyme, COX-2 is not expressed typically [23].

However, stimulation of various cells such as endothelial cells, monocytes, macrophages, vascular smooth muscle cells and fibroblasts by pro-inflammatory cytokines leads to the expression of COX-2 [23]. Up-regulation of COX-2 expression is associated with the secretion of inflammatory cytokines which enhance inflammation and tissue damage [23]. Moreover, COX-2 overexpression promotes proliferation of cells and disrupts equilibrium between apoptosis and cell proliferation [24]. In the current study, plumericin treatment of liver cancer cells inhibited the expression of COX-2.

The microenvironment of tumor cells consists of growth factors and chemotactic agents which promote tumorigenesis by increasing cell proliferation, migration and invasion [25]. The present study showed that plumericin exposure markedly reduced the invasion potential of Hep 3B cells. Studies have revealed that growth of liver cancer is reduced by some chemotherapeutic agents through targeting of the expressions of inflammatory cytokines [26,27]. Results from the present study revealed that plumericin treatment markedly inhibited tumor growth *in vivo* in a mice model, relative to untreated control mice.

CONCLUSION

The results obtained in this investigation indicate that plumericin efficiently suppresses liver cancer cell growth *in vitro* and *in vivo* through a mechanism involving the down-regulation of the expressions of COX-2 and VEGF. Thus, plumericin may be suitable for the clinical treatment of liver cancer.

DECLARATIONS

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Conflict of interest

The authors declare that no conflict of interest is associated with this study.

Authors' contribution

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. Liang Li designed the study and compiled the data. Jie Min, Lili Cao and Jun Zhou carried out the experiments and performed the literature study. All the authors wrote and approved the article for publication.

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