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Original Research Article

Isoalantolactone inhibits the proliferation of human liver cancer cells by inducing intrinsic apoptosis

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Abstract

Purpose: *To investigate isoalantolactone's potential as an anticancer agent targeting liver cancer cells and to elucidate the underlying mechanism.*
Methods: *Cell counting kit-8 (CCK-8) and colony formation assays were employed to analyze the anti-growth effects of isoalantolactone against liver cancer cells. Cell apoptosis was studied using Acridine orange/ethidium bromide (AO/EB) and Annexin V-FITC/Propidium iodide (PI) staining methods. The intracellular levels of reactive oxygen species (ROS) were estimated using the 2'-7'-Dichlorodihydrofluorescein diacetate (DCF-DA) method. Liver cancer cell invasion was assessed through Transwell assays.*
Results: *Isoalantolactone inhibited the proliferation and colony formation of HuH7 cells by inducing apoptosis. Isoalantolactone showed IC_{50} of 9 µM against HuH7 liver cancer cells. The MRC-5 normal cells treated with isoalantolactone also showed loss of viability and the IC_{50} was estimated to be 40 µM. HuH7 cancer cells administered with isoalantolactone exhibited modulation of apoptotic marker protein expression levels. Apoptosis was shown to result from ROS elevation. Isoalantolactone also restricted liver cancer cell invasion.*
Conclusion: *Isoalantolactone shows anti-proliferative and anti-metastatic effects against liver cancer cells via ROS-mediated apoptosis induction thereby making it a potential source of potent therapeutic agents against human cancer.*

Keywords: *Liver cancer, Sesquiterpenoid, Isoalantolactone, Metastasis, Apoptosis, ROS*

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INTRODUCTION

Liver cancer is one of the most frequently reported human cancers and is considered the fifth most common human cancerous malignancy [1]. In terms of total morbidity and mortality resulting from different human cancers per year, this malignancy is at present considered the third most lethal human cancer [2]. The 5-year survival rate for liver cancer is approximately 18 percent on average [3]. However, in the advanced stages of liver cancer, the disease becomes highly lethal [4]. Metastasis in lung tissues is frequent and among the dominant

causes of liver cancer-based death [5]. The currently applied treatment approach against liver cancer, including surgery combined with chemotherapy or radiotherapy, exhibits limited clinical success. Hence, research investigations are continuously focusing on evaluating the anticancer potential of different natural products to be used as lead molecules for devising more reliable therapeutic procedures against human liver cancer.

Evidence is accumulating regarding the therapeutic potential of plant-based products against a wide array of human diseases. These compounds have also demonstrated significant anti-tumor properties against various human cancer types [6,7]. Being the largest and most diverse type of plant secondary metabolites, terpenoids represent an ideal class for the identification of potent therapeutic agents against human cancer. Isoalantolactone is a sesquiterpenoid biosynthesized in several medicinal plant species which include *Inula spp*, *Radix inulae,* and *Aucklandia lappa* [8]. Isoalantolactone is known to exhibit anti-microbial, anti-helminthic, anti-fungal and anti-proliferative potential [9]. The anti-tumor effects of isoalantolactone have already been reported against some human cancer cells such as pancreatic, esophageal and lung cancer [10-12]. Taking a lead from this, isoalantolactone was examined for its anti-proliferative effect against human liver cancer cells in this study.

EXPERIMENTAL

Cell culture

Normal human liver fibroblast (MRC-5) and HuH7 liver cancer cell lines were procured from the Institute for Biological Sciences at the Chinese Academy of Sciences in Shanghai, China. Cell propagation was conducted using DMEM (Gibco) containing 10 % FBS (Hyclone). The cells were nurtured and kept in a humid incubation chamber at a temperature of 37 °C, supplemented with 5 % CO_2.

Cell counting kit-8 (CCK-8) viability and colony formation assays

The viability of HuH7 cancer cells and MRC-5 normal cells, subjected to various concentrations of isoalantolactone, was assessed using CCK-8 assay kit (Dojindo Laboratories). For the analysis of colony formation, HuH7 cells (10^4 cells/mL), treated with 4.5, 9.0, or 18 µM isoalantolactone, were cultured for 16 days in DMEM. The medium was replaced on alternate days. Following a 16-day interval, the medium was aspirated, followed by a thorough PBS rinse of cells. Then, 0.1 % crystal violet (Sigma-Aldrich) was used for staining the colonies for visualization and manual counting.

AO/EB and Annexin V-FITC/PI staining assays

HuH7 cells (10^5 cells/mL) were cultured in plates and incubated with 0, 4.5, 9, and 18 µM isoalantolactone for 24 h. Cells were collected and incubated with 200 µL of AO/EB staining mix (1:1) to stain the cells and visualize them under a fluorescent microscope (Olympus). On the other hand, HuH7 cells, subjected to varying isoalantolactone treatments, were subjected to staining using Annexin V-FITC apoptosis determination kit (Sigma-Aldrich). Cells were incubated with binding buffer (400 µL) at 25 ^oC for 15 min. Following this, a 20 µL combination of Annexin V-FITC and PI (1:1 ratio) was applied for a 20-minute staining period at room temperature (RT). Analysis of cell apoptosis was performed using a FACSCalibur flow cytometer (Becton Dickinson, NJ, USA).

Western blotting

For protein extraction, HuH7 cells subjected to various isoalantolactone treatments were lysed using cell lysis buffer (Thermo Fisher Scientific). Bradford's reagent was employed to determine protein concentrations. Exactly 40 µg proteins were resolved on SDS-PAGE gel and then blotted to PVDF membranes. The transferred membrane was probed using primary antibody: anti-Bcl-2 antibody (Abcam, ab196495; 1:800 dilution, USA), anti-Bax antibody (Abcam, ab53154; 1:1000 dilution, USA), and β-actin (Abcam, ab8227; 1:500 dilution, USA). Following the washing step, horse radish peroxidase (HRP) conjugated anti-rabbit, IgG secondary antibody (CST, 7074 1:4000 dilution, USA) was used and it was determined using the ECL chemiluminescence method.

ROS estimation by DCF-DA method

Dichlorodihydro fluorescein diacetate (DCF-DA) method was used for estimating the intracellular ROS levels. Briefly, HuH7 cells were treated with 0, 4.5, 9, or 18 µM isoalantolactone for 24 h at 37 ^oC. The cells were incubated with or without the ROS scavenger, Ascorbic acid (10 µM). Thereafter, the cells were harvested and washed with PBS and suspended in 100 µM DCF-DA for 25 min at 37 °C. Afterwards, the cells were lysed using alkaline solution. Finally, stained cells were examined under a fluorescent microscope.

Transwell assays

Migration and invasion of HuH7 cells, subjected to varying concentrations of isoalantolactone (0, 4.5, 9.0, or 18 µM), were assessed using Transwell chambers. Cell invasion was studied on a Transwell plate with a membrane filter having 8-µm pores. To determine the cell invasion, 250 µL of the medium carrying a suspension of 2.5 × 10^5 cells were seeded into the top chamber of Transwell plate coated with Matrigel. On the other hand, 650 µL of culture medium with 10 % FBS was added into the lower chamber. The cells were incubated for 24 hours at 37 ^oC. Then, the cells invading the lower chamber through the intervening membrane were fixed and stained using 0.2 % crystal violet solution. The number of invading cells was counted using five random field views under a light microscope (Olympus) and the percentage was determined.

Statistical analysis

Results of three independent experiments are recorded as mean ± standard deviation (SD). Student's *t*-test or one-way analysis of variance (ANOVA) was conducted to compare two experimental groups using GraphPad Prism version 7.0 software. A *p*-value < 0.05 was taken as a measure of statistically significant difference.

RESULTS

Isoalantolactone inhibits HuH7 cell viability

Liver cancer (HuH7) cells as well as normal fibroblasts (MRC-5) were incubated with sesquiterpene isoalantolactone (0 - 160 µM; Figure 1 A). Liver cancer cells (HuH7) exhibited a concentration-dependent loss in viability when treated with isoalantolactone, with an IC_{50} value of 9 µM (Figure 1 B). Normal cells (MRC5) on the other hand also showed loss of viability following treatment with isoalantolactone, but the IC_{50} was estimated to be 40 µM (Figure 1 C). This showed selective anti-proliferative action of isoalantolactone against liver cancer cells. To further confirm this result, HuH7 cells were exposed to 9 µM isoalantolactone for different periods. The findings indicated that the viability of HuH7 cancer cells declined with increasing isoalantolactone treatment durations (Figure 1 D). The anti-proliferative effects of isoalantolactone were also visualized in terms of concentration-dependent decline in HuH7 colony formation (Figure 1 E).

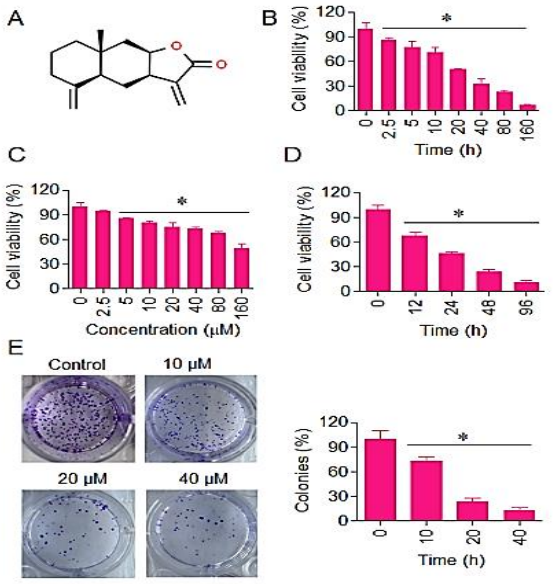

Figure 1: Isoalantolactone inhibits liver cancer cell viability and colony formation. (A) Structure of isoalantolactone. (B) Isoalantolactone reduces viability of HuH7 liver cancer cells with an estimated IC_{50} of 9 µM (C) Viability of MRC5 normal liver fibroblast cells were comparatively, minimally affected by different isoalantolactone concentration, with an estimated IC_{50} of 40 µM (D) Isoalantolactone-treated HuH7 cancer cells exhibited time specific decrease in rate of viability (E) Isoalantolactone inhibited colony formation from HuH7 cells; *$p < 0.05$

Isoalantolactone induces apoptosis in HuH7 cells

To determine isoalantolactone's anti-proliferative impact on liver cancer cells, HuH7 cancer cells were incubated with varying doses of isoalantolactone. The treated cells were then stained with AO/EB. With increasing isoalantolactone concentrations, the relative number of ethidium bromide-stained cells was shown to increase, indicative of induction of apoptosis (Figure 2 A). The cells were also stained with Annexin V-FITC/PI mixture. Flow cytometry showed that apoptotic HuH7 cells increased with an increase in isoalantolactone concentration (Figure 2 B). In addition, isoalantolactone up-regulated Bax and downregulated Bcl-2 (Figure 2 C).

Liver cancer cell viability was reduced by isoalantolactone through ROS elevation

Liver cancer (HuH7) cells were incubated with various doses of isoalantolactone for 24 h and stained with DCF-DA fluorescein to study intracellular ROS levels. The cells exhibited higher dichlorofluorescein (DCF) production as reflected by fluorescent microscopy (Figure 3 A). Dichlorofluorescein is produced as a

consequence of oxidation of DCF-DA by reactive oxygen species (ROS). Isoalantolactone therefore elevated the ROS levels (Figure 3 A). As expected, the incubation of isoalantolactone-treated HuH7 cells with ROS-scavenger, ascorbic acid, resulted in a decreased green fluorescence, further confirming the ROS elevation by isoalantolactone (Figure 3 B). Moreover, incubation of treated HuH7 cells with 10 µM ascorbic acid attenuated the anti-proliferative effects of isoalantolactone (Figure 3 B). The results thus showed that isoalantolactone elevated intracellular ROS levels to inhibit liver cancer cell growth.

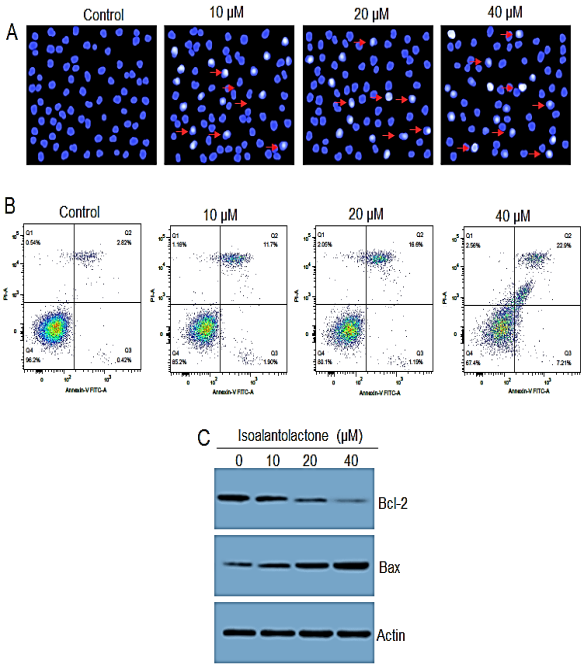

Figure 2: Isoalantolactone-induced apoptosis in HuH7 cells. (A) The relative percentage of HuH7 cells stained with ethidium bromide increased with increasing isoalantolactone concentrations as revealed by AO/EB staining (B) Increasing isoalantolactone concentrations increased the rate of HuH7 cell apoptosis (C) Western blot analysis showed that increase in isoalantolactone concentration changes the expression of marker proteins of apoptosis in HuH7 cells. The experiments were performed in triplicates. β-actin was used as a reference protein in western blotting

Isoalantolactone inhibits the invasion of liver cancer cells

Results from the Transwell assays showed that isoalantolactone significantly inhibited the invasion of HuH7 cells *in vitro* ($p < 0.05$) and in a manner contingent on concentration (Figure 4). Invasion decreased by more than 75 % when 18 µM isoalantolactone doses were used.

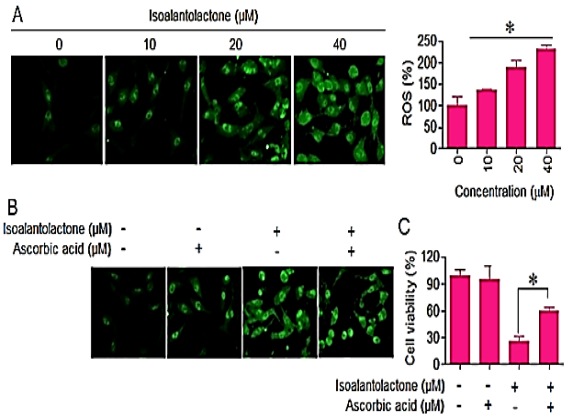

Figure 3: Isoalantolactone elevated intracellular ROS levels to decrease liver cancer cell viability. (A) Isoalantolactone increased ROS levels in HuH7 cells in a concentration-dependent manner as evidenced by an increase in dichlorofluorescein (DCF) fluorescence (B) The ROS elevation in HuH7 cells was minimized in the presence of ascorbic acid (C) Ascorbic acid attenuated the inhibition of viability by isoalantolactone, indicative of ROS-mediated induction of apoptosis in HuH7 cells; *$p < 0.05$

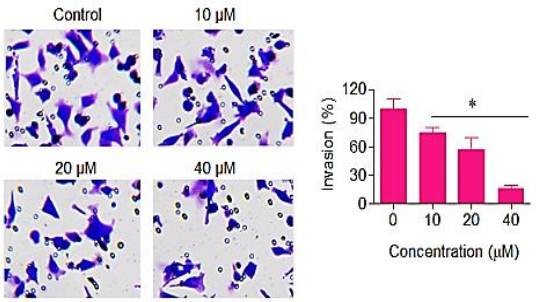

Figure 4: Isoalantolactone restricted liver cancer cell invasion *in vitro*. Transwell chamber assays showed that HuH7 cells exhibited a concentration-dependent reduction in their invasion *in vitro* once exposed to isoalantolactone; *$p < 0.05$

DISCUSSION

Natural products are indispensable and efficient molecules for drug discovery. There is growing evidence about their beneficial health effects and ability to prevent the onset of different human disorders including cancer [13]. Anticancer effects of natural products primarily rely on their capacity to induce apoptosis [14,15]. Isoalantolactone is effective in restricting the proliferation of several types of human cancer cells via the induction of apoptosis [16]. In this study, isoalantolactone treatment showed a concentration-dependent inhibition of liver cancer cell proliferation *in vitro.* The inhibition was a result of apoptotic cell death via modulation of the expression levels of apoptotic proteins. The apoptotic-inducing impact of isoalantolactone

aligns with the observations made by Chen *et al* [17] who reported apoptotic induction in prostate cancer cells by isoalantolactone.

Isoalantolactone was also found to modulate the expression of key marker proteins of apoptosis in liver cancer cells. The Bcl-2 family proteins are the crucial mediators of cell apoptosis [18]. The increase in Bax/Bcl-2 protein ratio could have acted as a vital cue for apoptosis onset in isoalantolactone-treated liver cancer cells.

Previous studies indicated that isoalantolactone-associated apoptotic cell death resulted from the elevation of intracellular ROS levels [17]. Herein, intracellular ROS was increased in the liver cancer cells treated with isoalantolactone. This indicated that apoptotic induction and subsequent loss of proliferation might have resulted from ROS elevation. The results were further confirmed by the finding that co-incubation of isoalantolactone-treated liver cancer cells with ascorbic acid, a scavenger of ROS, considerably attenuated the anti-proliferative effects of isoalantolactone. Transwell assay showed that isoalantolactone was effective in restricting the invasion of liver cancer cells, which further supports its anti-metastatic potential against liver cancer. Local and distant metastasis is the most dominant therapeutic hindrance to liver cancer management. The anti-metastatic potential of isoalantolactone is thus indicative of its possible therapeutic advantage against this serious and deadly human malignancy.

CONCLUSION

Isoalantolactone enhances intracellular ROS levels, leading to apoptosis and subsequently reducing cell viability. Additionally, *in vitro* experiments indicate that isoalantolactone treatment effectively restricts the invasion of liver cancer cells, further substantiating its capacity as an anticancer agent. These results pave the way for potential future applications of isoalantolactone in liver cancer therapy. This research underscores its promise as a novel liver cancer therapeutic, although further clinical investigations are necessary before translation into practical treatments.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

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

Conflict of Interest

No conflict of interest associated with 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. This manuscript was drafted by Wen Peng and Xin-geng Hui. The experiments were performed by Lin Huo, Dong-xiao and Zhi-cong Wu. The materials were collected by Ying Zhang, Xiao-bing Li, Tian Ma and Wen-hui Li. The statistical analysis was done by Jing Liang and Zhi-qiang Sun.

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