Antiproliferative effects of isosaltolactone in human liver cancer cells are mediated through caspase-dependent apoptosis, ROS generation, suppression of cell migration and invasion and targeting Ras/Raf/MEK signalling pathway

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The main objective of this study was to evaluate the in vitro antiproliferative effects of isosaltolactone against liver cancer cells (Hep-G2) and also monitor its mechanism of action. The MTT assay was involved in proliferation assessments and phase contrast microscopy was used to check cellular morphology. Acidine orange/ethidium bromide staining along with western blotting was used to evaluate proapoptotic effects of isosaltolactone. DCFH-DA staining was used in ROS measurements. Transwell migration and invasion assay were executed to check the effects of isosaltolactone on migration and invasion of Hep-G2 cells. Western blotting was used to check the expressions of Ras/Raf/MEK signalling pathway in Hep-G2 cells. Results demonstrated that isosaltolactone significantly (*p<0.05 and **p<0.01) inhibited the proliferation of Hep-G2 cells in a concentration and time-reliant fashion. The IC₅₀ value of the tested isosaltolactone molecule was found to be 71.2 µM and 53.4 µM at 12 h and 24 h time intervals respectively. Moreover, the antiproliferative effects of isosaltolactone were mediated through induction of caspase-dependent apoptosis and oxidative stress (ROS mediated). The proapoptotic effects of isosaltolactone were evident from morphological assessments and improved expressions of caspase-3, -8, and -9 and Bax while antiapoptotic Bcl-2 was reduced significantly. Additionally, antiproliferative and proapoptotic effects of isosaltolactone were found to be a consequence of blocking of Ras/Raf/MEK signalling in Hep-G2 cells. Furthermore, isosaltolactone significantly (*p<0.05) targeted the migration and invasion of Hep-G2 cells. In conclusion, these results validated that isosaltolactone shows strong antiproliferative activity against Hep-G2 liver cancer cells. Therefore, it could prove as a leading candidate in liver cancer research, drug discovery and design.

Keywords: Liver cancer, isosaltolactone, apoptosis, caspase, Ras/Raf/MEK signalling

INTRODUCTION

Liver malignancies are at global rise owing to adopted modern ways of life style (Zhen et al., 2017). Liver cancer is one among the predominant primary liver malignancies accountable for effecting an enormous human population each year. In the year of 2018, over 0.8 M new cases and 0.7 M deaths were registered due to liver cancer and ranked fourth in terms of mortality and sixth in terms of occurrence, worldwide (Bray et al., 2018). The risk factors found associated to the development of liver cancer include hepatitis B and C virus infections, cirrhosis, alcoholism, biliary cholangitis and non-alcoholic steatohepatitis (Anwanwan et al., 2020; Méndez-Sánchez et al., 2020). Contrast enhanced imaging is often used in the diagnosis of this disease (Wang et al., 2020). The understanding of carcinogenesis and staging of liver cancer has moved very fast. What once was perceived to be an orphan disease with no palliative and curative options, is now an area of immense research interest. Scientists have achieved significant breakthroughs in the field of liver cancer nevertheless to improve clinical outcomes of liver cancer a multidisciplinary approach is required globally.

Apoptosis is a programmed cell death and a primary pathological character of several liver disorders originating from different etiological factors including viruses, drugs, cholestasis and alcoholics (Wang & Lin, 2013). Hepatic apoptosis plays a vital role in maintaining the normal functioning of liver cells by elimination of malfunctioning once (Fabregat, 2009). The depletion of hepatic apoptosis is often responsible for worsening of liver functions leading to cirrhosis/fibrosis and tumorigenesis. Hepatic apoptosis proceed through extrinsic or intrinsic pathway stimulated by external or internal cell signalling, respectively (Bai & Odin, 2003). Intrinsic pathway involves the discharge of cytochrome c from mitochondrial membrane into cytoplasm where it activates caspase-9 while extrinsic pathway involves death receptor signals stimulating activation of caspase-8 and ultimately apoptosis (Molpeceres et al., 2007). Bringing about hepatic apoptosis in malfunctioning liver cells plays a vital role in suppression of lethal liver disorders including cancer.

Liver cancer carcinogenesis involves a number of cell signalling pathways and Ras/Raf/MEK signalling is one among them (Abou-Alfa, 2004). This pathway is an extracellular signal-regulated kinase (ERK) signalling often implicated in liver cancer development. The Ras/Raf/
MEK signalling gets activated on signals transmitted by growth resulting in the modulation of gene expressions and stimulation of transcription factors (Li et al., 2016). This signalling plays a key modulatory role in a number of vital cell functioning mechanisms including tumorigenesis (Guo et al., 2018). The Ras/Raf/MEK signalling is often found elevated in liver cancer cells.

The current study was designed to estimate the anti-liver cancer effects of isovalantolactone molecule. Isovalantolactone (Fig. 1a) is a member of active sesquiterpene lactones and has been found biologically important with diverse therapeutic potential (Rasul et al., 2013). The key source of isovalantolactone molecule is *Inula racemosa* (Liu et al., 2001). This plant is of remarkable pharmacological importance demonstrating significant anticancer effects against hepatocellular carcinoma, non-small cell lung cancer and fibrosarcoma (Ma et al., 2013). Isovalantolactone molecule has been reported to induce antiapoptotic effects against a variety of human cancers by inducing apoptosis including squamous cell carcinomas (head and neck), pancreatic cancer, prostate cancer, gastric adenocarcinoma and lung carcinoma (Rasul et al., 2013; Khan et al., 2012; Wu et al., 2013). In addition to proapoptotic effects, isovalantolactone has been recognized to target different cancer cell regulatory pathways including PI3K signalling, Wnt signalling and p38 MAPK/NF-κB signalling pathways (Zhang et al., 2021; Wang et al., 2016). The anticancer effects of isovalantolactone against liver cancer have not yet been fully evaluated and understood. Therefore, in this study we evaluated the anti-liver cancer effects of isovalantolactone alongside investigated its mechanism of action against Hep-G2 cells.

**MATERIALS AND METHODS**

**Chemicals, reagents and cell culture**

The test molecule isovalantolactone (>98% purity by HPLC) was procured from Tauto Biotech, Shanghai, China. All the chemicals and reagents involved in this study, unless otherwise mentioned, were obtained from Merk KGaA (Darmstadt, Germany). The target cell line Hep-G2 was obtained from Type Culture Collection of Chinese Academy of Science (Shanghai, China). Cultural medium RMPI-1640 was obtained from Hyclone, Logan, UT and 10% FBS from ThermoFisher Scientific Inc. (Waltham, USA). The Hep-G2 cells were maintained in RMPI-1640 medium bearing penicillin G and streptomycin under humid environment of 37°C in a 5% CO₂ incubator.

**Proliferation assay**

We investigated the effects of isovalantolactone on proliferation of Hep-G2 cells considering MTT assay. In brief, Hep-G2 cells of concentration 1×10⁵ cells/well in 96-well plates were precultured for 24 h. Cells were harvested and subjected to treatment with isovalantolactone for 12 h and 24 h at different concentrations viz 0, 25, 50, 75 and 150 µM. Following isovalantolactone exposure, the MTT reagent (20 µL) was supplied to the cells and then incubated for 4 h at 37°C. Post reagent treatment cells were loaded with DMSO (150 µL) to dissolve the formazan crystals. Finally, colorimetric analysis was performed by taking absorbance at 570 nm using a micro-plate reader (Varioskan Flash, Thermo Scientific). The production of formazan is directly proportional to viable cells and each experiment was repeated in triplicates.

**Phase-contrast microscopy**

To examine morphological changes induced by isovalantolactone to Hep-G2 cells, treated and control cells were visualized through phase-contrast microscopy. In brief, Hep-G2 cells at logarithmic growth phase were harvested and plated uniformly to 24-well plates with 1.4×10⁴ cells/well. For attachment, cells were placed under incubation for 24 h at 37°C. Post attachment period, cells were treated with different isovalantolactone concentrations of 0, 25, 75 and 150 µM following by 48 h of incubation. After being treated with isovalantolactone, Hep-G2 cells subjected to morphological evaluation under a phase contrast microscope (Leica, Wetzlar, Germany).

**Acridine orange/ethidium bromide (AO/EB) staining**

We investigated proapoptotic effects of isovalantolactone against Hep-G2 cells via AO/EB staining. The dual AO/EB fluorescent staining is detected under a fluorescent microscope to identify apoptosis-allied changes in cells under investigation for apoptosis. In brief, a concentration of 1.2×10⁵ cells/well of Hep-G2 cells were plated to 24-well plate. These well plates were exposed to different isovalantolactone concentrations viz 0, 25, 75 and 150 µM. Treated cells were placed under incubation at 37°C for 48 h followed by the addition of 4 µg/mL of AO/EB staining solution and left for 5 min. Post staining of isovalantolactone treated Hep-G2 cells, cells were instantaneously directed to fluorescence microscopy using a fluorescent microscope (Nikon Corporation, Tokyo, Japan).

**Flow cytometry**

The DCFH-DA staining aided by flow cytometry was used to monitor ROS formation in isovalantolactone treated Hep-G2 cells. Concisely, a concentration of 1.2×10⁵ cells/well of Hep-G2 cells were plated to 6-well plate and left to attach in RMPI-1640 growth medium overnight. Post attachment, cells were exposed to isovalantolactone using concentrations of viz 25, 75 and 15 µM for 24 h. Control group received DMSO. Following test molecule treatment, Hep-G2 cells were
stained using DCFH-DA (10 µM) Beyotime, Shanghai, China. Post staining, Hep-G2 cells were placed under incubated for half an hour at 37°C in absence of light. As a final point, cells were collected for fluorescence assessment to check ROS through flow cytometry using FACSCalibur flow cytometer (BD, Franklin Lakes, NJ).

**Transwell migration assay**

Transwell chambers (Corning Coaster, United States) were utilized for the assessment of effects of isoalantolactone on migration of Hep-G2 cells. In brief, transwells were placed with RPMI-1640 medium containing 0.1% bovine serum albumin (Gibco, Life Technologies) and lower transwells were filled with serum-free medium and 5% fetal bovine serum as chemo-attractant. Hep-G2 cells (2×10⁶) were treated with different concentrations of isoalantolactone for 48 h viz 0, 25, 75 and 150 µM, followed by immediate transference to upper chambers. After transfer, transwells were incubated for 24 h at 37°C and then non-migrated cells were cleared using a cotton swab. Migrated cells were subjected to fixing, staining and washing using ethanol, crystal violet (0.1%) and PBS, respectively. Finally, the migrated cells were pictured followed by further analysis under a microscope.

**Transwell invasion assay**

A similar protocol to that of transwell migration assay was adopted to monitor effects of isoalantolactone on invasion of Hep-G2 cells. Except, here we used bovine serum albumin free medium and transwells were coated with Matrigel (BD, Pharmingen).

**Western blotting**

The Hep-G2 cells were plated in 6-well plate at a concentration of 4×10⁴ cells/well followed by overnight settlement before exposure to different isoalantolactone concentrations viz 0, 25, 75 and 150 µM for 48 h. Post treatment, Hep-G2 cells were lysed using RIPA lysis buffer and protein content was estimated by BCA assay. Carefully, 40 µg of proteins were separated over sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10%) and then moved to PVDF membranes. Subsequently, the membranes were blocked with skimmed milk in TBST (5%) for 1 h at 37°C. Then the membranes were left with primary antibodies overnight at 4°C using antibodies against caspase-3, -8 and -9, Bax, Bcl-2, Ras, Raf and MEK (Santa Cruz Biotechnology, Shanghai, China). Thereafter, membranes were placed with HRP-conjugated monoclonal secondary antibodies at room temperature for 1 h. Finally, the bands of proteins were developed using enhanced chemiluminescence and pictured.

**Statistical analysis**

All the data from individual triplicate experiments were shown as mean ± S.D. Data analysis were performed using GraphPad prism software version 5 and comparisons were executed using one-way ANOVA and then Dunnett’s test. The values of *p*<0.05 and **p**<0.01 were used to reveal statistically significant differences.

**RESULTS**

**Isoalantolactone inhibited proliferation of Hep-G2 cells**

The antiproliferative feature of isoalantolactone against Hep-G2 cells was evaluated by MTT assay. Treatment with isoalantolactone (0–150 µM) inhibited the proliferation of Hep-G2 cells significantly. The inhibition in proliferation showed time as well as concentration-dependence. In comparison to the control group (*p*<0.05), treatment groups (**p**<0.01) showed significant reduction in viability percentage, after 12 h and 24 h of isoalantolactone exposure. On higher concentrations (150 µM), isoalantolactone reduced viability of Hep-G2 cells to almost 20% and 5%, respectively (Fig. 1b). The IC₅₀ value of the tested isoalantolactone molecule was found to be 71.2 µM and 53.4 µM at 12 h and 24 h time intervals respectively.

**Isoalantolactone disrupted morphology**

In morphological assessment, phase contrast microscopy was performed and the results demonstrated that attached cells grew well for control group. No evident morphological variation was seen in control cells, but the isoalantolactone treated group (25 µM) revealed apparent changes in normal morphology and structure like cell floating, appearance of reduced density and round detached cells. At higher isoalantolactone concentrations (75 and 150 µM), the morphology of Hep-G2 cells turned irregular, which characterized apoptosis like apoptotic bodies, membrane shrinkage, blebbing, fragmentation and condensation of chromatin (Fig. 2). Therefore, these results of morphological assessment indicated that isoalantolactone could induce apoptotic cell death.

**Isoalantolactone induced apoptotic death**

We estimated proapoptotic effects of isoalantolactone against Hep-G2 cells through AO/EB labeling and evaluated using fluorescence microscope. No apparent apoptosis was observed in control group. The apoptotic cells were marked in treatment groups by orange-red or red fluorescence (Fig. 3). On increasing the isoalantolactone concentrations (75–150 µM), the number of cells indicating apoptotic cell death became increased. AO/EB

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**Figure 2. Phase-contrast microscopy results showing characteristic proapoptotic features of Hep-G2 cells post isoalantolactone treatment.**

The pictures shows cell floating, cell detachment, apoptotic bodies, membrane shrinkage, blebbing, fragmentation and condensation of chromatin in treated group compared to controls. Individual experiments were repeated three times.
staining showed that treatment group revealed structural disintegration and nuclear damage. Furthermore, western blotting presented improved levels of expressions of caspases (3, 8 and 9) and Bax, in isoalantolactone treated group against control group (Fig. 4). The levels of antiapoptotic Bcl-2 proteins were significantly blunted by isoalantolactone in treated group compared to controls. Therefore, these results confirm that antiproliferative effects of isoalantolactone follow caspase-dependent proapoptotic pathway.

Isoalantolactone augmented ROS generation

Intracellular ROS play crucial role in cell death and carcinogenesis. We herein demonstrated the effects of isoalantolactone on intracellular ROS production against Hep-G2 cells. For that we performed DCFH-DH staining followed by flow cytometric analysis. Results demonstrated that isoalantolactone elevated the production of ROS in Hep-G2 cells (*p<0.05) while no apparent rise was seen in control group treated with DMSO. The elevation of ROS production followed a concentration dependent pattern in treated Hep-G2 cells. The ROS percentage was increased by almost four folds (Fig. 5) on increasing the isoalantolactone concentrations from 25–150 µM. Therefore, these results showed that isoalantolactone induced oxidative stress allied antiproliferative effects in Hep-G2 cells.

Isoalantolactone inhibited cell migration and cell invasion

Cell migration and invasion in treated/control Hep-G2 cells were evaluated through transwell assay. The pictures of the Hep-G2 cells pre/post isoalantolactone treatment showed significant deduction in cell migration (Fig. 6a). The number of migrated cells was inversely proportional

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**Figure 3.** The AO/EB staining assay was used to monitor apoptosis in Hep-G2 cells post isoalantolactone treatment. The results indicate irregular morphology of Hep-G2 cells with increased orange-red and red fluorescence with increasing isoalantolactone concentrations. Individual experiments were repeated three times.

**Figure 4.** Western blotting analysis showing the expression bands of caspase-3, 8, and 9, Bax and Bcl-2 in treated and control Hep-G2 cells. Individual experiments were repeated three times.

**Figure 5.** Flow cytometric analysis of DCFH-DA staining showing ROS production in treated/control Hep-G2 cells. The graph clearly indicates increase in ROS production with increased doses of isoalantolactone. Data are shown as mean ± S.D. of individually performed triplicate experiments. (*p<0.05)

**Figure 6.** (a) Pictures representing the migrated Hep-G2 cells in control/treatment groups. (b) The quantitative analysis of cell migration assay and the results clearly indicate isoalantolactone inhibit migration of Hep-G2 cells. Data are shown as mean ± S.D. of individually performed triplicate experiments. (*p<0.05)

**Figure 7.** (a) Pictures representing the invasive Hep-G2 cells in control/treatment groups. (b) The quantitative analysis of cell migration assay and the results clearly indicate isoalantolactone inhibit invasiveness of Hep-G2 cells. Data are shown as mean ± S.D. of individually performed triplicate experiments. (*p<0.05)
to that of isoalantolactone concentrations (Fig. 6b). Control group showed no signs of reduction in migration while treated group (25–150 µM) showed a significant decrease (p<0.05). Similar results were obtained in case of invasion analysis. Isoalantolactone inhibited the invasiveness of Hep-G2 cells following concentration-reliant pattern. The number of invasive cells in control group was almost 470, which decreased to almost 100 indicative of retardation of cell invasion (Fig. 7a and b).

**Isoalantolactone blocked Ras/Raf/MEK signalling**

The Ras/Raf/MEK signalling in Hep-G2 cells treated/untreated cells was checked by western blotting. The results declared that isoalantolactone exhibits inhibitory potential on Ras/Raf/MEK signalling pathway. The expression levels of Ras and Raf were found significantly lower in treated groups when compared to controls. The expressions of these proteins lowered in a concentration-reliant fashion. Moreover, no apparent change was noticed in the expression bands of t-MEK while significant decrease was observed in case of p-MEK. This indicated that isoalantolactone inhibited the phosphorylation process of MEK in cancerous Hep-G2 cells (Fig. 8). These results validated that isoalantolactone inhibited phosphorylation and blocked the expressions of Ras/Raf/MEK signalling in Hep-G2 cells compared to controls.

**DISCUSSION**

Despite the advances in understanding of liver cancer carcinogenesis and treatment, the clinical outcome of presently available methodologies remain poor. This generates a need for novel and efficient treatment methodologies including chemotherapeutics. Natural products have served humanity in numerous ways including drug design and discovery (Anwar et al., 2020). They are a huge reservoir of chemical entities showing immense structural diversities. Yet more discoveries are to come as we have only studied fewer of natural products maximum remain untraced. Sesquiterpene lactones are a class of bioactive natural products showing rich pharmacological profile (Adekenov, 2017). They have been shown to possess significant properties of antioxidants, anti-inflammatory, antiviral, antibacterial, analgesic, antitumor, antipryptic and anticancer (Choudhary & Mishra, 2019).

Isoalantolactone is one of the strong anticancer agents of this class of compounds. In this study we found that isoalantolactone inhibited the proliferation of Hep-G2 liver cancer cells in a dose-reliant fashion mediated via induction of caspase-dependent apoptosis, oxidative stress, and suppression of Ras/Raf/MEK signalling. Moreover, we found that isoalantolactone could inhibit the metastatic liver cancer by inhibiting the migration and invasion abilities of Hep-G2 cells.

Several studies have reported antiproliferative effects of isoalantolactone against a variety of human cancers including the cancers of pancreas, stomach and lung (Rasul et al., 2013; Khan et al., 2012; Wu et al., 2013). Isoalantolactone have been shown to induce apoptosis against a wide range of human cancer cell lines including SK-MES-1, DU145 and PC-3 cells (Jin et al., 2017; Chen et al., 2018). It has been shown to up-regulate Bak and down-regulate Bcl-2 in lung cancer cells along with cell cycle arrest. In this study, we found that isoalantolactone induced antiproliferative effects in Hep-G2 cells via promotion of apoptosis. The proapoptotic nature of isoalantolactone was further evident by its up regulatory effects on proapoptotic proteins like Bax, caspase-3, -8 and -9. Therefore, our results showed consistency with the previously reported once were isoalantolactone induced apoptosis allied anticancer effects. As hepatic apoptosis plays a key role in the regulation of normal functioning and carcinogenesis of liver cells, isoalantolactone could prove beneficial in overcoming this lethal malignancy (Fabregat, 2009).

Reactive oxygen species (ROS) are the key agents that trigger chromosomal alterations and genetic mutations which lead to the occurrence of fatal doses like cancer (Liou & Storz, 2010). ROS interfere with several cell survival mechanisms at different steps resulting in premature execution and ultimately carcinogenesis. Normally the ROS production is checked and counterbalanced by redox molecules and antioxidant enzymes (Hunyadi, 2019). ROS maintenance is important for maintaining normal redox state in cells. Moderate enhancement in ROS could induce cell growth and proliferation while acute and abrupt increase in ROS could lead into cell death or uncontrolled proliferation (Pelicano et al., 2004). Therefore, ROS are one of the prominent targets of chemotherapeutics in cancer cells to stimulate death. Isoalantolactone has been previously shown to inhibit prostate cancer via induction ROS-mediated ER-oxidative stress (Chen et al., 2018). We herein found that isoalantolactone significantly up regulated the ROS production in Hep-G2 cells in a dose-reliant fashion. Therefore, over results showed consistency with the previously published studies showing ROS enhancing effects of isoalantolactone.

Cell migration and invasion play vital role in liver cancer metastasis (Yang et al., 2013). Therefore, targeting migration and invasion in liver cancer cells is one of the efficient strategies to overcome metastatic liver cancer. Isoalantolactone has been previously reported to induce inhibitory effects over migration and invasion of breast cancer cells (Wang et al., 2016). It targeted p38 and MAPK/NF-κB signalling of in MDA-MB-231 cells thereby inhibited migration and invasion. Herein, we found that isoalantolactone showed potential inhibition of migration and invasion in Hep-G2 cells. Thus, isoalantolactone could prove as a lead in management of metastatic liver cancer.

We first time demonstrated the effects of isoalantolactone on Ras/Raf/MEK signalling pathway in liver cancer Hep-G2 cells. This pathway over activation in liver leads to carcinogenesis and hence could be utilized as a potential target in suppression of liver cancer. Results showed isoalantolactone inhibited the Ras/Raf/MEK signalling in Hep-G2 cells significantly.
CONCLUSION

The results of this study suggested that isoalantolactone could prove as a potential drug candidate against liver cancer. Isoalantolactone induced dose-dependent proliferation inhibitory effects against liver cancer HepG2 cells via promotion of caspase-dependent apoptosis, inducing oxidative stress, inhibiting cell migration and cell invasion and blocking of Ras/Raf/MEK signalling. Therefore, we recommend further in vivo and clinical studies to demonstrate anticancer efficacy in liver of isoalantolactone molecule.

Conflict of interest

The authors have no conflict of interest to declare.

REFERENCES


