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ORIGINAL ARTICLE

Growth inhibition of Saos-2 osteosarcoma cells by lactucopicrin is mediated *via* inhibition of cell migration and invasion, sub-G1 cell cycle disruption, apoptosis induction and Raf signalling pathway

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Summary

Purpose: *Lactucopicrin, a sesquiterpene lactone, has been reported to exhibit anticancer activity against different cancer types. In this study, the anticancer effect of Lactucopicrin was examined against human osteosarcoma cells along with its effects on cell migration and invasion, cell cycle phase distribution and Raf signalling pathway.*

Methods: *The human osteosarcoma cells Sao-2 were treated with various concentrations of Lactucopicrin for 24 h. The anti-proliferative effects of Lactucopicrin were measured by CCK8 cell viability assay. Acridine orange (AO)/ ethidium bromide (EB) and annexin V/propidium iodide (PI) assays were employed to examine the induction of apoptosis. Transwell assay was performed to examine the cell migration and invasion. Protein expression analysis was performed by western blot analysis.*

Results: *Lactucopicrin inhibited the proliferation of Saos-2 cells and exhibited an IC_{50} of 25 μM. The antiproliferative effects were due to induction of apoptosis as indicated by AO/EB staining. Moreover, the annexin V/PI staining showed that the percentage of the apoptotic cells increased with increase in the concentration of Lactucopicrin. The induction of apoptosis was also related to upregulation of Bax and downregulation of Bcl-2. Lactopucrin also caused arrest of the osteosarcoma cells at the sub-G1 phase of the cell cycle. Transwell assay showed that Lactucopicrin inhibited the migration and invasion of the Saos-2 cells. Finally, Lactucopicrin also blocked the Raf signalling pathway in the Saos-2 cells in a concentration-dependent manner.*

Conclusions: *Lactucopicrin exhibits significant antiproliferative effects on the osteosarcoma cells and may prove essential in the development of systemic therapy for this malignancy.*

Key words: *lactucopicrin, osteosarcoma, apoptosis, cell cycle arrest, invasion*

Introduction

Osteosarcoma is a malignant bone tumor threatening the life of children and adolescents. It is characterized as highly aggressive type of cancer and pulmonary metastases is the major cause for death [1]. Many modern and advanced medications have been used to enhance the survival of osteosarcoma patients but the effectiveness of the currently available treatments are very limited because of drug resistance and relapses [2]. Studies reported that about 30% of osteosarcomas are multidrug-resistant [3]. Many reports established a relationship between the fast bone growth during the development of osteosarcoma and pubescence. It has been shown that 56% of all the osteosarcomas are found around the knees [4]. Therefore, the need of the hour is to look for new and po-

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tent anticancer agents for the management of this malignancy. Plant-derived Sesquiterpene lactones (SLs) have shown impressive promise as anticancer agents. The growth inhibition of different types of cancer by SLs has been reported by several authors [5]. These metabolites are prevalently found in the Asteraceae family of plants [6]. Many of the SLs have even reached clinical trials [7]. Lactucopicrin is an important SL and has recently been shown to inhibit the growth of skin cancer cells *via* multiple mechanisms [8]. Nonetheless, the anticancer effects of Lactucopicrin have not been examined against the human osteosarcoma cells. This study was therefore designed to investigate the anticancer effects of Lactucopicrin against the human Saos-2 osteosarcoma cells together with investigation of the underlying mechanisms. The main focus of this study was to examine and analyse the growth inhibitory effects of lactucopicrin on Saos-2 human osteosarcoma cells along with evaluating its effects on cellular migration and invasion, sub-G1 cell cycle phase distribution, apoptosis and Raf signalling pathway.

Methods

Cell viability determination

The CCK-8 assay was used for the determination of the cell viability of osteosarcoma cells. In brief, the Saos-2 cells were seeded in 96-well plates and treated with varied concentrations of Lactucopicrin at 37°C for 24 h. Thereafter, 10 µL of CCK-8 solution were added to the cell culture and incubated for 2 h at 37°C in a humidifier (5% CO_2, 95% O_2). Optical density $(OD)_{450}$ was taken with a microplate reader to determine the cell viability.

AO/EB and Annexin V/PI staining assay

The Saos-2 cells (0.6×10^6) were seeded in 6-well plates and exposed to varied concentrations of Lactucopicrin for 24 h at 37°C. Ten µl cell cultures were put onto glass slides and stained with a solution of AO/EB. The slides were cover-slipped and assessed by fluorescent microscopy. Annexin V/PI staining was performed as described previously [8].

Cell cycle analysis

The Saos-2 cells were treated with varied concentrations of Lactucopicrin and cultured for 24 h at 37°C. Phosphate buffered saline (PBS) was then used to wash the cells harvested by centrifugation. The cells were then stained with PI and the cell distribution was evaluated by flow cytometry.

Transwell assay

The effects of Lactucopicrin on the invasion ability of Saos-2 cells was determined by transwell chambers (8 mm pore size, Corning, NY, USA) with Matrigel (Millipore, Billerica, USA) The Saos -2 cells were cultured and 200 ml cell cultures were placed onto the upper chamber and only the RPMI 1640 medium was placed in the bottom chamber. After 24 h of incubation, the cells were removed from the upper chamber and the cells that invaded *via* the chamber were fixed with methyl alcohol and subsequently stained with crystal violet. Inverted microscope was used to count the number of invaded cells at 200X magnification.

Western blot analysis

The determination of the protein expression was carried out by western blotting. The Lactucopicrin-treated Saos-2 cells were harvested with centrifugation. The Saos-2 cells were then lysed in lysis buffer containing protease inhibitor. Estimation of protein content within each lysate was performed *via* BCA protein assay kit (Beyotime Institute of Biotechnology, China). Afterwards, using SDS-PAGE (10 %) uniform protein amounts (35 µg) were separated and loaded over PVDF membranes bought from Millipore, Billerica, MA, USA. Next, fat-free milk was used to block the membrane at room temperature for 1 h. Afterwards, the membranes were treated with primary antibodies at 4°C overnight. Subsequently, the membranes were incubated with secondary antibodies. Finally, protein bands were visualized through enhanced chemiluminescence (ECL) detection system (Pierce, Rockford, IL, United States).

Statistics

The results are presented as values from three independent experiments with the data expressed as means ± standard deviation. Differences between the groups were examined by Student's t-test using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

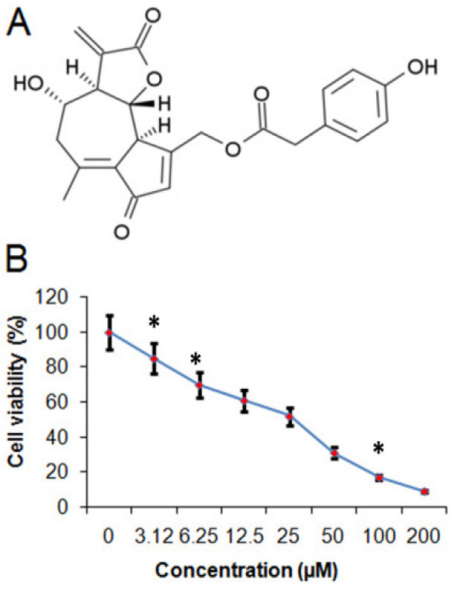

Figure 1. (A): Chemical structure of Lactucopicrin. **(B):** Effect of Lactucopicrin on the viability of Saos-2 cells as determined by CCK8 assay. The values are mean of three experiments ± SD (*p<0.05).

Results

Effect of Lactucopicrin on the viability of osteosarcoma cells

The CCK-8 assay was used to unveil the effects of Lactucopicrin on the viability of the Saos-2 osteosarcoma cells (Figure 1A). Lactucopicrin caused a significant reduction in the viability of the Saos-2 cells which was dose-dependent and IC_{50} of 25 μM was observed for Lactucopicrin against the Saos-2 cells (Figure 1B).

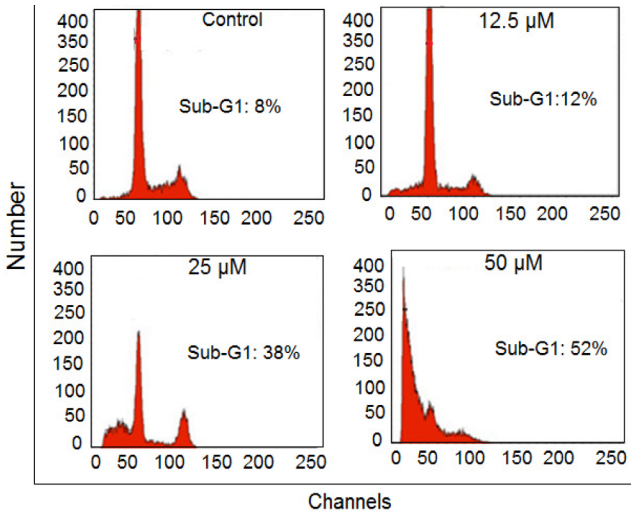

Figure 2. Effect of Lactucopicrin on the distribution of the Saos-2 cells in different cell cycle phases at indicated concentrations. The experiments were performed in triplicate. Lactucopicrin led to dose-dependent induction of sub-G1 cell cycle arrest, the percentage of sub-G1 cells increased from 8% at 0 μM dose to 52% at 50 μM dose.

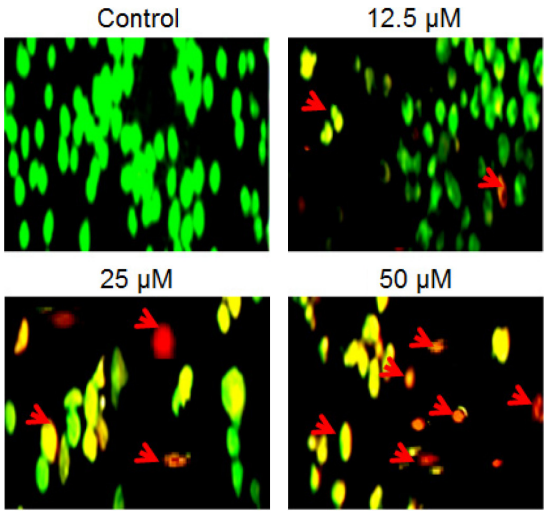

Figure 3. Lactucopicrin induces apoptosis in Saos-2 cells as indicated by AO/EB staining. The experiments were performed in triplicate. In AO/EB staining assay, yellow and orange fluorescence indicates apoptotic cells which are shown here by arrows, note the significant increase in apoptotic cells with increasing dose of Lactucopicrin.

Lactucopicrin causes arrest of the Saos-2 cells at the Sub-G1 checkpoint

The effects of Lactucopicrin were also evaluated on the distribution of the Saos-2 cells in cell cycle phases. The results indicated that Lactucopicrin treatment caused a significant increase in the sub-G1 phase of the cell cycle, indicative of sub-G1 arrest (Figure 2). The percentage of the sub-G1 phase cells increased from 8% in untreated cells (0 μM dose) to 52% at 50 μM concentration of Lactucopicrin.

Apoptotic effects of Lactucopicrin on osteosarcoma cells

To ascertain the underlying mechanism for the growth inhibitory property of Lactucopicrin, the Saos-2 cells were treated with different doses of this compound and then stained with AO/EB. The results of AO/EB assay showed that Lactucopicrin caused nuclear fragmentation of the Saos-2 cells, characteristic of apoptosis (Figure 3). Annexin V/PI staining showed that the apoptotic cell percentage increased from 2.14% in control to 24.34% at 50 μM concentration of lactucopicrin (Figure 4). Western blot analysis showed that Lactucopicrin caused increase in the Bax and decrease in the Bcl-2 expression, confirming the apoptotic cell death in the Saos-2 cells (Figure 5).

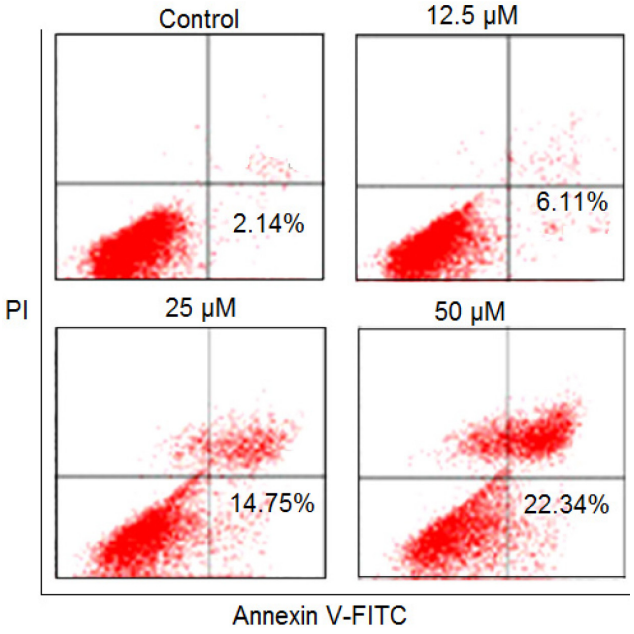

Figure 4. Determination of the percentage of the apoptotic Saos-2 cell populations as determined by Annexin V/PI staining. The experiments were performed in triplicate. Annexin V/PI staining assay is used to quantify the percentage of apoptotic cells which increased with increasing doses of Lactucopicrin.

Lactucopicrin inhibits the migration and invasion of the Saos-2 cells

Transwell assay was used to monitor the effect of different concentrations of Lactucopicrin on the migration and invasion of the Saos-2 cells. The results showed that Lactucopicrin caused significant decrease in the migration (Figure 6) and invasion (Figure 7) of the Saos-2 cells. These effects of Lactucopicrin on the cell migration and invasion were found to be concentration-dependent.

Lactucopicrin deactivates the Raf pathway in the Saos-2 cells

The effects of Lactucopicrin were also investigated on the phosphorylation status of the Raf in the Saos-2 cells (Figure 8). The results showed that the phosphorylation of Raf decreased concentration-dependently upon treatment with Lactucopicrin with no apparent effects on the total Raf.

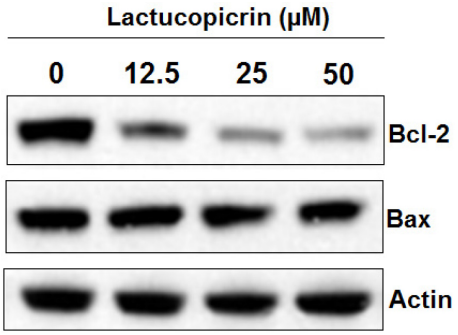

Figure 5. Effect of Lactucopicrin on the expression of Bax and Bcl-2 expression in Saos-2 cells as indicated by western blot analysis. The experiments were performed in triplicate. Lactucopicrin led to significant and dose-dependent increase in the expression of Bax and a decrease in the expression of Bcl-2 proteins indicating that this molecule induces apoptosis.

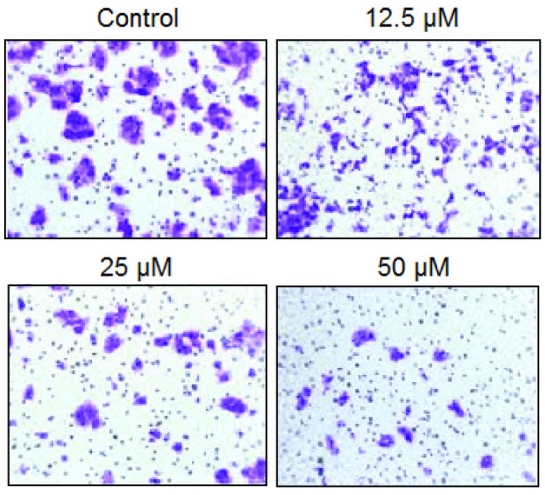

Figure 6. Inhibition of Saos-2 cell migration by Lactucopicrin at indicated concentrations as depicted by transwell assay. The values are mean of three experiments. Lactucopicrin led to dose-dependent inhibition of cell migration of Saos-2 osteosarcoma cells.

Discussion

Osteosarcoma causes remarkable mortality worldwide and the treatments for this disease are not efficient and are associated with a number of adverse effects [9]. It is therefore urgent to look for new efficient therapeutic agents that could be employed in the treatment of osteosarcoma. Herein, we examined the anticancer effects of Lactucopicrin on the Saos-2 osteosarcoma cells. The results revealed that Lactucopicrin inhibits the growth of the osteosarcoma cells in a concentration-dependent manner and also halted their ability to grow further. Previous studies have shown that Lactucopicrin could inhibit the growth of several types of cancers such as skin cancer [8,10]. Moreover, it

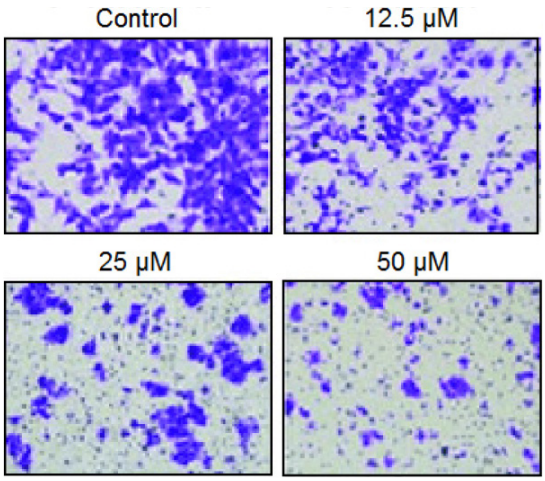

Figure 7. Inhibition of Saos-2 cell invasion by Lactucopicrin at indicated concentrations as depicted by transwell assay. Lactucopicrin led to dose-dependent inhibition of cell invasion of Saos-2 osteosarcoma cells. The values are mean of three experiments.

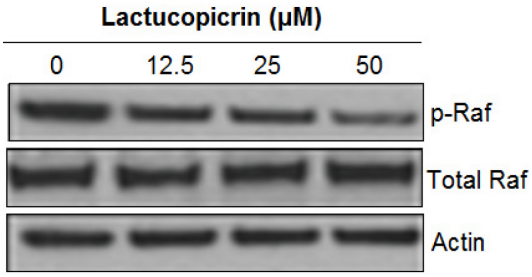

Figure 8. Inhibition of the phosphorylation of Raf by Lactucopicrin at indicated concentrations as depicted by western blot analysis. The experiments were performed in triplicate. The results showed that the phosphorylation of Raf decreased concentration-dependently upon treatment with Lactucopicrin with no apparent effects on the total Raf.

has also been shown that Lactucopicrin induces apoptosis in cancer cells, for example Lactucopicrin has been reported to induce apoptosis in skin cancer cells [8]. Therefore, we investigated whether Lactucopicrin also induces apoptosis in the Saos-2 osteosarcoma cells. The results of AO/EB and annexin V/PI showed that Lactucopicrin induced apoptosis in the Saos -2 cells and the percentage of the apoptotic cells increased in parallel with increase of the concentration of Lactucopicrin. Apoptosis is an important process that helps eliminate the harmful and cancer cells and several known anticancer drugs induce apoptosis [11]. The Lactucopicrin-induced apoptosis was also associated with concomitant increase in the expression of Bax and downregulation of Bcl-2 which are the important markers for apoptosis [12]. Many of the plant-derived anticancer agents have also been shown to cause cell cycle arrest of cancer cells [13]. Herein, we observed that Lactucopicrin caused arrest of the osteosarcoma cells at the sub-G1 phase of the cell cycle.

Osteosarcoma cells have the capacity to invade distant tissues and develop metastasis [14] and hence we assessed the effect of Lactucopicrin on the migration and invasion of the Saos-2 cells. Interestingly, it was found that Lactucopicrin could suppress the migration and invasion of Saos-2 cells with concomitant downregulation of MMP-2, 3 and 9 expression. STAT3 transduction pathway is an important pathway that has been reported to be dysregulated in cancer cells [19] and in this study we found that Lactucopicrin inhibited the phosphorylation of the Saos-2 osteosarcoma cells.

Conclusion

It is concluded that Lactucopicrin exhibits remarkable anticancer effects on the osteosarcoma cells *via* induction of apoptosis and cell cycle arrest. In addition, Lactucopicrin also inhibited the migration and invasion of osteosarcoma cells. Therefore Lactucopicrin may prove beneficial in the therapeutic management of osteosarcoma.

Conflict of interests

The authors declare no conflict of interests.

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