

ORIGINAL ARTICLE

Royleanone diterpenoid exhibits potent anticancer effects in LNCaP human prostate carcinoma cells by inducing mitochondrial mediated apoptosis, cell cycle arrest, suppression of cell migration and downregulation of mTOR/PI3K/AKT signalling pathway

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Summary

Purpose: Prostate cancer is a deadly malignancy and is responsible for significant cancer-related mortality in men. The incidence of prostate cancer is continuously increasing across the globe and the existing treatment options for this disease are limited and associated with a lot of side effects. Therefore there is an urgent need to identify novel and efficient therapeutic agents for the management of prostate cancer. In the current study we evaluated the anticancer activity of royleanone diterpenoid against prostate cancer LNCaP cell line.

Methods: The anticancer activity of royleanone was determined by CCK8 assay. Apoptosis was detected by acridine orange and ethidium bromide as well as by annexin V/propidium iodide (PI) staining. Mitochondrial membrane potential (MMP) and cell cycle analysis were investigated by flow cytometry and protein expression by western blotting.

Results: The results showed that royleanone exerts potent anticancer activity on LNCaP prostate cancer cells with an IC_{50} of 12.5 μ M at 48 hrs of incubation. The anticancer activity of royleanone was due to induction of cell cycle arrest and mitochondrial-mediated apoptosis. Moreover, royleanone could also suppress the cell migration potential and inhibited the mTOR/PI3/AKT signalling pathway in LNCaP prostate cancer cells.

Conclusions: Taken together, we propose that royleanone could prove to be an important anticancer lead molecule for the treatment and overall management of prostate cancer, provided further in vivo studies are carried out.

Key words: apoptosis, cell cycle arrest, prostate cancer, royleanone

Introduction

Prostate cancer is one of the most prevalent cancers in men which increasing incidence around the world. The screening and diagnosis of prostate cancer and subsequent treatment is challenging and existing treatments are limited [1]. It has been reported that more than 40,000 men die of prostate cancer in USA annually, being the second ma-

jor cause of cancer-related mortality in males [2]. Even if surgical interventions and/or radiotherapy may be curative for localized prostate cancers, the advanced-stage prostate cancer is associated with a poor prognosis. Moreover, current hormonotherapy is less effective and therefore the shortage of treatment options for prostate cancer demands

development of novel therapies for its treatment [3,4]. Natural products have displayed a diversity of health promoting properties since immemorable times. Natural products are mainly secondary metabolites synthesized by living organisms to adapt to and survive under extreme environmental conditions. Owing to the tendency of these secondary metabolites to interact with enzymes, receptors and other cellular entities, they exhibit significant drug properties. Reports from 1981 to 2010 have revealed that a number of natural products were established as sources of new drugs [5]. Furthermore, the diverse molecular scaffolds of natural products often display drug properties [6], and are considered essential for combinatorial [7,8] and diversity-oriented synthesis [9] for the development of effective bioactive molecules.

Over the years plant-derived diterpenoids have emerged as an important class of anticancer agents. The anticancer activity of these diterpenoids have been attributed to their potential to induce accretion of reactive oxygen species (ROS), alteration of MMP, apoptosis and cell cycle arrest. A number of diterpenoids has been evaluated *in vitro*, *in vivo* and even in clinical trials for their anticancer activity [10]. Royleanone (Figure 1) is an important diterpenoid isolated from several plant species and has been shown to exhibit impressive pharmacological potential [11]. However, the anticancer activity of Royleanone has not been investigated against prostate cancer so far. In the current study we investigated the anticancer activity of royleanone diterpenoid against the prostate cancer LNCaP cell line.

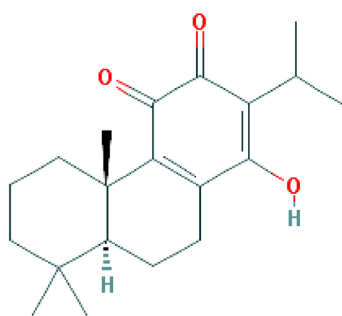


Figure 1. Chemical structure of royleanone.

Methods

Chemicals, reagents and cell culture

Royleanone and other chemicals were of reagent grade and purchased from Sigma Chemical Co. (St. Louis, Missouri, USA) unless otherwise mentioned. The human prostate cancer cell line (LNCaP) was procured from Type Culture Collection of Chinese Academy of Sciences, Shanghai, China. The cells were cultured in RPMI-1640

medium containing 10% fetal bovine serum (FBS), 100U/mL penicillin and 100µg/mL streptomycin and maintained in a humidified atmosphere containing 5% CO₂.

CCK8 assay for assessment of cell viability

The cell viability of the prostate LNCaP cancer cell line was determined by CCK8 assay. Briefly, 5×10^5 cells were placed in a 96-well plate and incubated at 37°C in a humidified, 5% CO₂ atmosphere. After overnight incubation the cells were treated with different concentrations of royleanone (0-200 µM) for 48 hrs. Thereafter, 10 µL of CCK8 were added into each well and cells were incubated again at 37 °C for 1 hr. The optical density (OD) at OD₄₅₀ nm was determined by a microplate spectrophotometer (BioRad, Segrate, Italy). The cell viability was presented as percent of the control.

Acridine orange (AO)/ethidium bromide (EB) and annexin V/PI staining

For the detection of apoptosis the LNCaP cells were cultured at the density of 2×10^5 cells/well in 6-well plates and treated with different concentrations of royleanone for 24 hrs. The cells were then stained with a mixture of AO and EB and were examined under fluorescent microscope. For annexin V/PI a procedure similar to AO and EB staining was followed except for the cells stained with annexin V/PI and investigated by flow cytometry.

Determination of mitochondrial membrane potential

To evaluate the MMP, LNCaP cells were seeded at a density of 2×10^5 cells/well in a 6-well plate and treated with 0, 6.25, 12.5 and 25 µM royleanone for 48 hrs at 37°C in 5 % CO₂ and 95% air. Afterwards, cells from all samples were collected, washed twice with phosphate buffered saline (PBS) and re-suspended in 500 µl of DiOC6 (1 µmol/l) for MMP examination at 37°C in the dark for 30 min. The samples were then examined instantly by flow cytometry.

Cell cycle analysis

The cell cycle analysis was carried out by flow cytometry after staining with PI. In brief, LNCaP prostate cancer cells were plated at a density of 2×10^5 , treated with different concentrations of (0, 6.25, 12.5 and 25 µM) and then were harvested and incubated with ethanol (70%) at 4°C. After overnight incubation the cells were collected again by centrifugation at 1000 rpm for 10 min and washed with PBS and finally resuspended in 250 ml of PBS and treated with RNase for 20 min. Afterwards, the cells were then stained with PI and cell cycle analysis was carried out by flow cytometry.

Cell migration assay

For cell migration of LNCaP, the wound healing assay was carried out. Briefly, cells were cultured till confluence (80%). Afterwards, the LNCaP cells were treated with 0, 6.25, 12.5 and 25 µM of royleanone and a scratch was made with a sterile pipette tip and the cells were incubated for 24 hrs. The wound healing capacity

of royleanone-treated cells was determined by comparing the wound length with that of the untreated control cells.

Protein expression by western blotting

Total protein from untreated and royleanone-treated cells was isolated in RIPA lysis buffer. Equal protein extracts from each group were run on SDS PAGE and then transferred to a polyvinylidene fluoride membrane. This was followed by blocking with 5% non-fat milk and incubation at room temperature for 1 hr. Thereafter, the membranes were incubated with a specific primary antibody at 4 °C overnight. This was followed by washing in washing buffer and incubation for 1 hr with the suitable secondary antibody. The protein bands of interest were visualised by an ECL Advanced Western Blot Detection Kit (GE Healthcare, Uppsala, Sweden).

Statistics

Data were expressed as mean \pm SD. Statistical significance was analyzed by Student's t test with the help of GraphPad Prism Demo, Version 5 (Graph Pad Software, San Diego, California, USA). Statistical significance was presented as * p < 0.05, ** p < 0.01 and *** p < 0.001.

Results

Royleanone exerts anticancer effects on LNCaP cells

The anticancer effect of royleanone against human prostate LNCaP cancer cells was evaluated by CCK8 assay. The results indicated that royleanone exerted dose-dependent antiproliferative effects on LNCaP cells (Figure 2). The IC_{50} of royleanone against LNCaP cells was found to be 12.5 μ M. Moreover, the anticancer effects of royleanone on LNCaP cancer cells exhibited a dose-dependent pattern.

Royleanone triggers mitochondrial apoptosis

AO/EB staining was carried out to investigate if royleanone triggered apoptosis in LNCaP cells. The

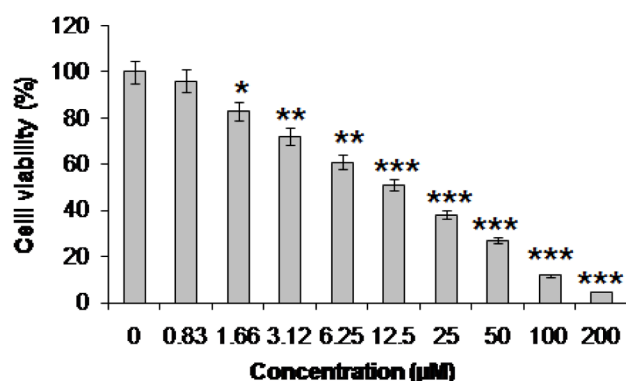


Figure 2. Assessment of cell viability of LNCaP prostate cancer cells by CCK8 assay. Experiments were carried out in triplicate and expressed as mean \pm SD. The values were considered significant at * p < 0.01, ** p < 0.001 and *** p < 0.0001.

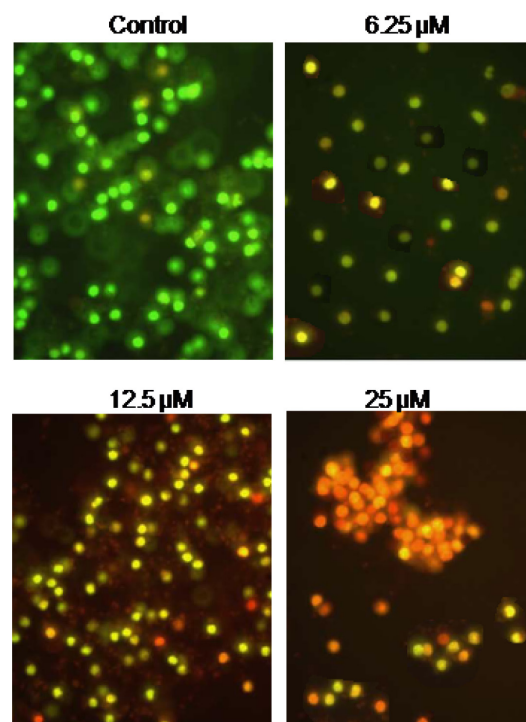


Figure 3. Assessment of apoptosis inducing potential of royleanone at the indicated doses in LNCaP cancer cells by AO/EB staining. The experiments were carried out in triplicate. The figure shows that royleanone induces apoptosis in LNCaP cells in a concentration-dependent manner.

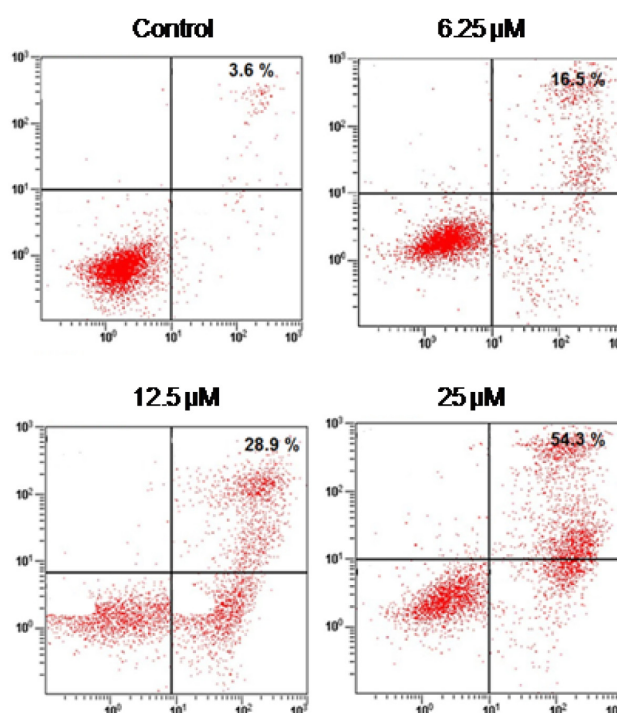


Figure 4. Annexin V/PI staining was carried out for the assessment of the apoptotic LNCaP prostate cancer cell populations at the indicated concentrations of royleanone. The experiments were carried out in triplicate. The figure depicts that the percentage of apoptotic cells increases concentration-dependently.

results indicated that royleanone induced apoptosis in LNCaP cells as evidenced from the increasing orange fluorescence in case AO/EB staining (Figure 3). To estimate the apoptotic cell populations, we carried out annexin V/PI staining which revealed that apoptotic cells increased with increasing concentrations of royleanone (Figure 4). The apoptotic cell populations were found to be 3.6, 16.5, 28.9 and 54.3% at the concentrations of 0, 6.25, 12.5 and 25 μM of royleanone. Next, we determined the effect of different concentrations of royleanone on MMP. The results indicated that royleanone treatment decreased the MMP of LNCaP cells in a dose-dependent manner (Figure 5). The inhibition of cell migration was also associated with the concomitant upregulation of Bax and downregulation of Bcl-2 expression (Figure 6).

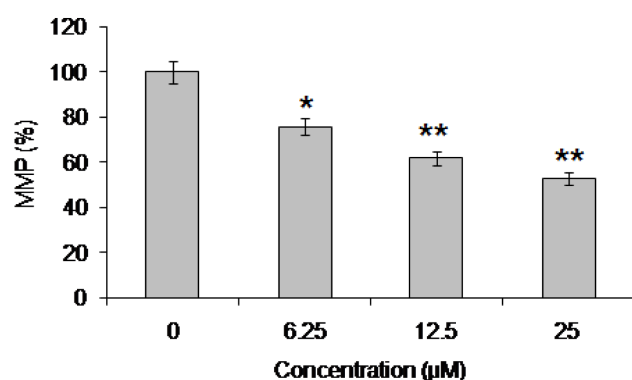


Figure 5. Determination of mitochondrial membrane potential in LNCaP cancer cells. Experiments were carried out in triplicate and expressed as mean \pm SD. The values were considered significant at * $p < 0.01$ and ** $p < 0.001$. The figure shows that MMP levels increases with increase in the concentration of royleanone.

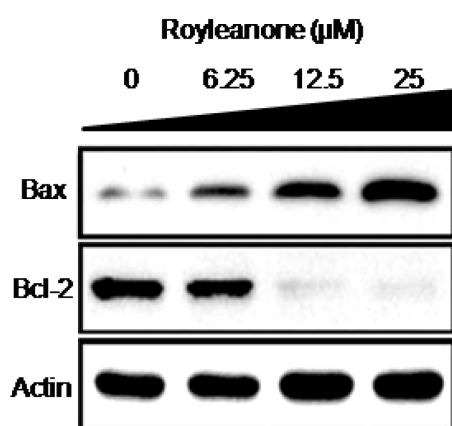


Figure 6. Examination of expression levels of apoptotic and anti-apoptotic proteins in LNCaP prostate cancer cells by western blotting. Experiments were carried out in triplicate and expressed as mean \pm SD. The figure depicts that Bax expression increases and the Bcl-2 expression decreases in LNCaP cells upon royleanone treatment.

Royleanone induces G0/G1 cell cycle arrest

Cell cycle arrest is one of the important mechanisms by which anticancer agents exert their inhibitory effects. Therefore, we also determined the effect of royleanone on cell cycle phase distribution of royleanone-treated LNCaP cells (Figure 7). Our results indicated that the percentage of LNCaP

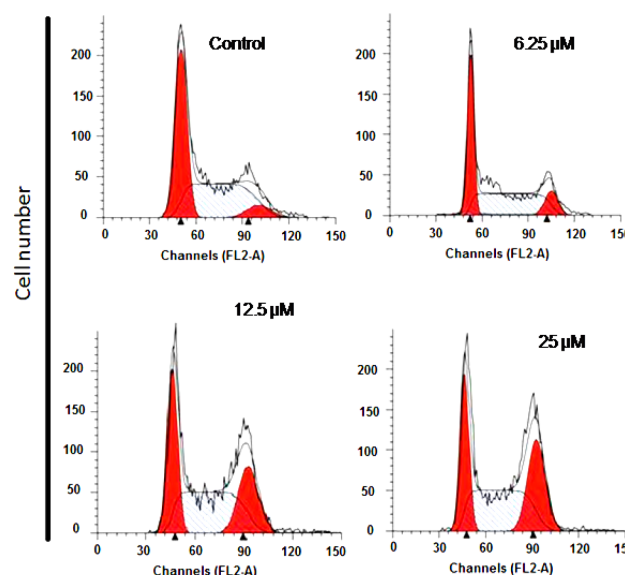


Figure 7. Effect of royleanone at indicated doses on the cell cycle phase distribution of LNCaP prostate cancer cells. The experiments were carried out in triplicate. The figure shows that royleanone induces G2/M cell cycle arrest of LNCaP cells.

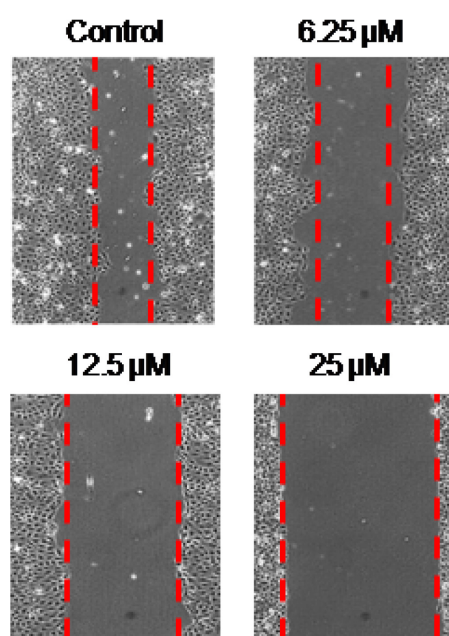


Figure 8. Effect of royleanone at indicated doses on the cell migration LNCaP prostate cancer cells by wound healing assay. The experiments were carried out in triplicate. The figure reveals that royleanone inhibits significantly the migration of the LNCaP cancer cells.

cells was considerably increased in G2 phase at the concentrations of 0 to 25 μM of royleanone, causing G2/M cell cycle phase arrest. Additionally, the populations of LNCaP cells in G2 phase were marginally increased at a dose of 6.25 μM , reasonably increased at 12.5 μM , and dramatically increased at 25 μM , indicating a dose-dependent effect of royleanone.

Royleanone inhibits cell migration

We further investigated whether royleanone could inhibit the migration of LNCaP cancer cells at different concentrations by wound healing assay. The results of this assay showed that royleanone reduced the migratory capability of LNCaP cells in a dose-dependent manner. However, the control the cells showed fairly good capacity to migrate as depicted in Figure 8.

Royleanone blocks mTOR/PI3K/AKT signalling pathway

The mTOR/PI3K/AKT signalling pathway has been reported to play important roles in tumorigenesis and progression of several cancers including prostate cancer. Therefore, we evaluated the effect of varied concentrations of royleanone on

this cascade. The results indicated that royleanone inhibited the phosphorylation of PI3K, mTOR and AKT in a dose-dependent manner (Figure 9).

Discussion

Prostate cancer is the second major cause of cancer-related mortality in men. It has been reported that in United States prostate cancer causes 40,000 deaths annually [2]. The treatment of prostate cancer mainly involves surgery, radiotherapy and/or hormonotherapy. However, the disease relapses and the results are far from satisfactory. Moreover, the hormonal and chemotherapeutic agents for prostate cancer are limited and create lots of side effects with significant impact on the patient quality of life [12]. Therefore, the current needs demand to look for more efficient and novel anticancer agents with potential for development of new systemic therapies for prostate cancer. Consistent with this, natural products have been considered as important sources of anticancer agents and several natural products have been screened for their anticancer activity. This has led to the development of several natural anticancer compounds that are currently being used for the treatment of a large diversity of cancers. In fact it has been reported that about half of the currently used anticancer drugs are of natural origin [6-8]. Among natural products, plant-derived diterpenoids have shown promising results. These diterpenoids have been evaluated against different cancer types *in vitro* and *in vivo* and even some have undergone clinical trials [12].

In the present study we evaluated the anticancer activity of royleanone diterpenoid against LNCaP prostate cancer cells. Our results revealed that royleanone exhibits potent anticancer activity against these cells with an IC_{50} of 12.5 μM . To further investigate the underlying mechanism of the anticancer activity of royleanone, we performed AO/EB staining which revealed that royleanone induces apoptosis in LNCaP cells in a dose-dependent manner. To further confirm the apoptosis-inducing potential of royleanone we carried out annexin V/PI staining which showed dose-dependent increase in the apoptotic cell populations. The results of the present study are also supported by earlier investigations wherein diterpenoids have been reported to induce apoptosis in cancer cells [13]. To find out if royleanone induced apoptosis via the mitochondrial pathway, we determined that the MMP caused dose-dependent reduction in the MMP which was associated with concomitant upregulation of Bax and downregulation of Bcl-2 expression, suggestive of mitochondrial-mediated apoptosis. Apart

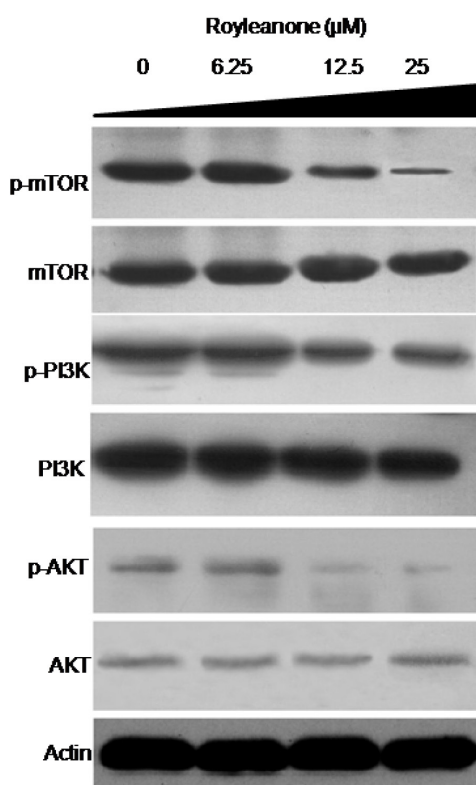


Figure 9. Effect of royleanone on the expression of key proteins of mTOR/PI3K/AKT signalling pathway by Western blotting. The experiments were carried out in triplicate. The figure shows that royleanone inhibits the mTOR/PI3K/AKT signalling pathway in prostate cancer cells.

from apoptosis, cell cycle arrest is another mechanism by which anticancer agents exert their effects. In our study we observed that royleanone induced G2/M cell cycle arrest in LNCaP cells in a dose-dependent manner. A previous study has reported that diterpenoids are able to induce cell cycle arrest of cancer cells [13]. Cell migration is the key feature of cancer progression and metastasis [14] and suppression of cell migration may prove essential in the inhibition of metastasis *in vivo*. This may ensure comparatively longer survival of patients [14]. In the present study it was observed that royleanone inhibited the migration of LNCaP cells in a dose-dependent manner. Finally, we investigated the effect of royleanone on the mTOR/PI3/AKT signalling pathway which is an important pathway involved in tumorigenesis and progression of different cancers. Our results showed that royleanone could inhibit the expression of some of the important proteins such as p-PI3K, p-mTOR and p-AKT.

Conclusion

From our results we conclude that royleanone is an important diterpenoid with very significant potential to inhibit the growth of prostate cancer cells. The anticancer effects of this compound are due to induction of apoptosis, cell cycle arrest and inhibition of mTOR/PI3K/AKT signalling pathway. We believe that royleanone could prove a potential lead molecule for the treatment of prostate cancer.

Acknowledgement

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Conflict of interests

The authors declare no conflict of interests.

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