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ORIGINAL ARTICLE

Lanostane inhibits the proliferation and bone metastasis of human breast cancer cells via inhibition of Rho-associated kinase signaling

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Summary

Purpose: *This study was performed to investigate the effects of lanostane against human breast cancer cells with emphasis on its potential to inhibit cancer cell growth and metastasis along with understanding the underlying molecular mechanism mediating the effects.*

Methods: *The SK-BR-3 normal breast line and the MB-157 breast cancer cell line were used in this study. MTT of cell growth was used to determine the viability of cells under lanostane treatment. Colony formation assay was used to analyze the clone forming capability of cancer cells when treated with lanostane. DAPI and acridine orange (AO)/ethidium bromide (EB) staining assays were performed for assessing the apoptic cell death. The level of cellular apoptosis was further examined using flow cytometry. Wound healing and transwell assays were performed to determine the migration and invasion of cancer cells. Western blotting was used for determining the concentration of proteins of interest.*

Results: *The lanostane treatment of cancer cells resulted in loss of cell viability. The IC_{50} value was 15μM and the inhibitory effects were dose-dependent. However, the inhibition of cell proliferation in normal breast cells was comparatively lower. The antiproliferative effects of lanostane were modulated through Bax/Bcl-2 pathway inducing apoptosis of cancer cells. Furthermore, the lanostane rendered cancer cells less motile and reduced their metastasis remarkably. The inhibition of cell metastasis was modulated through Rho-associated kinases (ROCK) signaling pathway which is involved in metastasis of breast cancer to bone tissues. Hence, the results suggested that lanostane inhibited the breast cancer metastasis to bone.*

Conclusion: *The results of the present study are suggestive of anticancer effects of lanostene triterpene which exerted its effects by inhibiting cell proliferation and metastasis of breast cancer cells mediated through inactivation of Rho-associated kinase signaling. The study holds promise to provide a lead for exploring the secondary metabolite-based anticancer approach against various human malignancies.*

Key words: *lanostane, metastasis, breast cancer, anticancer, cell proliferation, secondary metabolite*

Introduction

Plants produce a vast number of chemical compounds through specialized sets of metabolic reactions constituting the secondary metabolism. The secondary metabolites are usually characteristic of a particular group of organisms [1]. Secondary metabolites, apart from being beneficial to host organ-

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isms themselves, they are seen to possess a variety of health promoting features. Terpenoids are the most dominant among the secondary metabolites and about 60% of all the secondary metabolites produced are terpenoids [2]. Terpenoids, with their antimicrobial potential, are used in combating some prominent human diseases like malaria [3,4]. The terpenoids also show promise to aid in the fight against cancer. There is a good number of reports about the anticancer effects of terpenoid compounds [5-8]. Prompted by the anticancer potential of terpenoids, here we tried to assess the effects of lanostane against human breast cancer. Lanostane (Figure 1A) is a triterpene and lanostane-type of triterpenoids have antiproliferative and cytotoxic effects [9,10]. Breast cancer is the second most frequently detected cancer in females in USA excluding skin cancer [11]. The overall mortality rate of breast cancer makes it the fifth most death-causing malignancy, worldwide. The mortality is due to metastasis to distant organs. Despite recent advances, 20-30% of early detected breast cancers metastasize [12]. Metastasis occurs usually to lung and bones [13]. Despite understanding the genetic basis of metastasis of breast cancer to underlying tissues is really complex, studies have shown that Rho GTPases play a crucial role in this process [14,15]. The Rho GTPases are mediated by the action of Rho-associated kinases (ROCKS). The ROCKS, with two isoforms ROCKI and ROCK II have been shown to regulate the breast cancer metastasis. The progression of breast cancer to advanced stages progresses with increasing ROCK signaling and inhibition of ROCK signaling enhances the overall survival rate [16]. In yet another study, the inactivation of ROCK signaling was shown to reduce the growth of breast cancer cells *in vitro* and inhibit the metastasis to bone *in vivo* [17]. The investigation of anticancer effects against breast cancer cells revealed that lanostane is active in inhibiting the growth of cancer cells in a concentration-dependent manner. The antiproliferative effects were evident as induction of cell apoptosis by lanostane mediated through Bax/Bcl-2 apoptotic signaling. In addition, the migration and invasion of cancer cells were negatively affected. The investigation of inhibition of metastasis showed that the inhibitory signal was mediated through inactivation of Rho-associated kinase signaling. The inhibition of Rho- Summing up, lanostane is active in restricting the growth of breast cancer cells by the induction of cancer cells. The key finding of the present study is that the cancer cell migration and invasion was significantly inhibited by lanostane treatment by inactivating the Rho-associated kinase signaling and thus revealed its potential to prevent cancer metastasis to bone tissues.

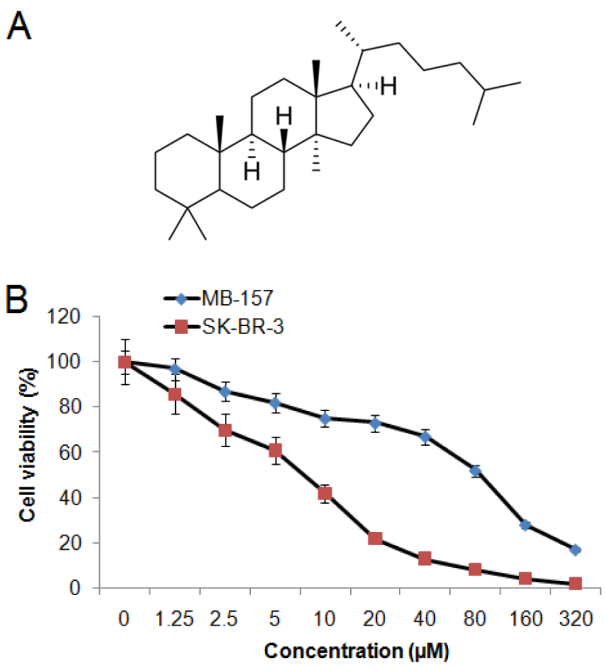

Figure 1. A: Chemical structure of Lanostane. **B:** Cell viability of normal MB-157 and breast cancer SK-BR-3 cells. The experiments were performed in triplicate and expressed as mean ± SD ($p < 0.05$).

Methods

Cell culture

The SK-BR-3 normal breast cell line and the MB-157 cancer cell line were purchased from American Tissue Culture Collection (ATCC, USA). The cell lines were cultured with Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 1µg streptomycin (Invitrogen) and 100 U/ml penicillin (Invitrogen). Humididified incubator was used to maintain the cell lines with 5% CO_2 at 37°C.

Determination of cell viability

Both SK-BR-3 and MB-157 cells were treated with 0, 1.25, 2.5, 5, 10, 20, 40, 80, 160, 320µM concentrations of lanostane for 24h. The cells were then seeded in 96-well plates at a density of 1×10^6 cells/well. To each well 0.5% MTT solution was added followed by incubation at 37°C for 4h. Then, 100µl of DMSO was added to each well for dissolving formazan crystals. The optical density (OD) was recorded for each sample using spectrophotometer at 570nm for determining the percent cell viability.

Cell viability of cancer cells treated with 0, 10, 20 and 40µM lanostane was also assessed using colony formation assay. The cells were seeded at a density of 1×10^6 cells/well in a 6-well plates followed by incubation with 5% CO_2 for 2 days at 37°C. After incubation, 4% paraformaldehyde was used for 20 min to fix the cells which were then stained using 0.1% crystal violet for 20 min.

Examination of apoptotic cell death

The cancer cells were treated with 0, 10, 20 and 40μM concentrations of lanostane for 24h and then seeded at a density of 2×10^5 cells/well in 96-well plates. This was followed by incubation of 24h at 37°C. The cells were then harvested, fixed with 70% ethanol for 20 min, stained with DAPI or AO/EB staining solutions. Slides were made and cells were analyzed for nuclear morphology under fluorescent microscope. The cells were also double-stained using Annexin V-FITC/PI and analyzed for the level of apoptosis using flow cytometry.

Cell migration and invasion assays

The migration of cancer cells treated with 0, 10, 20 and 40μM lanostane was assessed using wound healing assay. In brief, culturing of cancer cells was done in 6-well plates. A scratch line was drawn on the cell surface with the tip of a sterile 200μl pipette. After 24h incubation at 37°C, the scratch was examined for assessment of cell migration.

The transwell assay was used to determine the invasion of MB-157 cancer cells treated with 0, 10, 20 and 40μM lanostane. Briefly, about 250μl of cancer cell suspension at a density of 4×10^5/ml were put into the upper chamber and only culture medium was kept in lower chamber. Then, 24h incubation at 37°C followed. The cells that invaded the lower chamber were fixed using ethanol and then stained with crystal violet and visualized using high magnification (200×).

Western blot analysis

Western blotting was performed for determining the concentration of different proteins using actin protein as control. The cancer cells were lysed using RIPA lysis buffer and cell lysates were assessed for total protein concentration with the Bradford's method. About 45μg of protein were loaded from each sample and then ran on SDS-PAGE which was put to PVDF membrane. The membrane was exposed to primary antibodies followed by exposure to secondary antibodies and finally the proteins of interest were visualized employing chemiluminescence reagent.

Statistics

The experiments of this study were performed in triplicate. The values are shown as average±standard deviation. One-way ANOVA and Student's *t*-test were performed using Graphpad Prism7.0 software. P value <0.05 was indicative of statistically significant difference.

Results

Effects of lanostane on the proliferation of cancer cells

When MB-157 cancer cells were treated with different doses of lanostane and assessed for determination of their proliferative rates, it was observed their proliferation was inhibited and the pattern of inhibition was concentration-dependent. The proliferation of cancer cells decreased by about a half at 15μM lanostane concentration which was taken as IC_{50} value. However, the antiproliferative effects of lanostane were much less against the SK-BR-3 normal breast cells (Figure 1B), showing selective anticancer activity. The growth inhibitory effects of lanostane were also evident as lesser clone forming potential of cancer cells treated with lanostane (Figure 2).

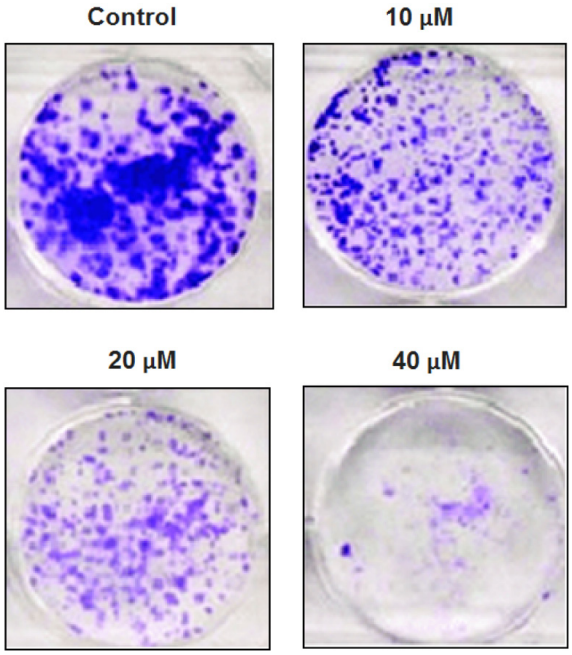

Figure 2. Colony formation of the lanostane-treated breast cancer SK-BR-3 cells. The experiments were performed in triplicate.

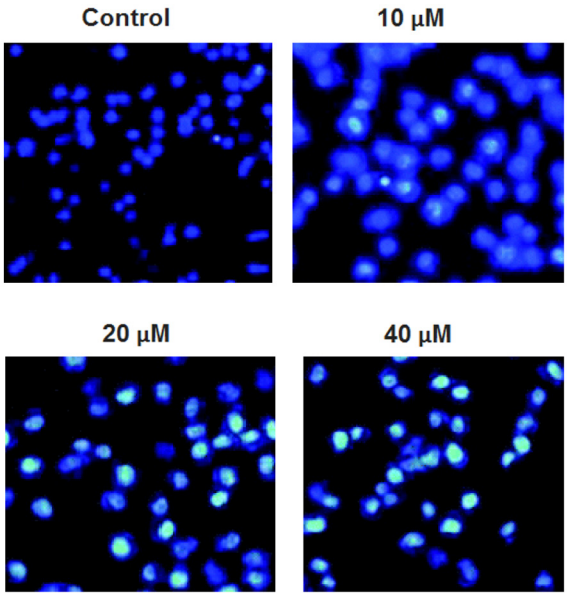

Figure 3. DAPI staining of the lanostane-treated breast cancer SK-BR-3 cells. The experiments were performed in triplicate.

Lanostane induced apoptosis in breast cancer cells

To analyze whether lanostane inhibited the cancer cell growth through induction of apoptosis, DAPI and AO/EB staining methods were used. DAPI examined for nuclear fluorescence showed clear deformation of chromatin indicative of cellular apoptosis (Figure 3). The AO/EB stained cancer cells also revealed the same results when examined under fluorescent microscope (Figure 4). The induction of cancer cell apoptosis under lanostane treatment was further proved by using flow cytometry which showed that the percentage of apoptotic cells increased with increase in lanostane concentration (Figure 5). To infer whether the apoptosis is induced through Bax/Bcl-2 apoptotic signaling, concentrations of Bax and Bcl-2 were determined under different lanostane doses which showed that Bax concentration increased while of Bcl-2 decreased with increasing concentrations of lanostane (Figure 6). The western blotting results confirmed that induction of apoptosis by lanostane was modulated via the Bax/Bcl-2 signaling pathway.

Lanostane inhibited metastasis of breast cancer cells in vitro

Cancer cells were treated with different doses of lanostane and their migration and invasion potential was assessed using wound healing and transwell assays, respectively. The cell migration was seen to be inhibited in a dose-dependent manner (Figure 7). Further, the invasion of cancer cells was also inhibited and the level of inhibition increased when higher concentrations of lanostane were used (Figure 8). The results suggest that lanostane decreased the mobility of cancer cells and thus can reduce the metastasis of breast cancer.

Lanostane-mediated inactivation of ROCK signaling and inhibition of bone metastasis

To understand the mechanism of inhibition of cell proliferation and metastasis of breast cancer cells by lanostane, its effects on the signaling components of ROCK signaling pathway, i.e., RhoA GTPase and ROCK II were analyzed. The results showed that lanostane was not having any effect on native RhoA and ROCK II proteins but the lev-

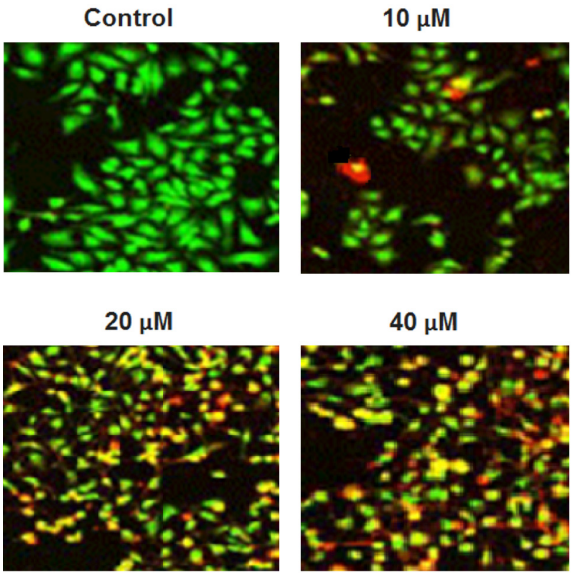

Figure 4. AO/EB staining of the lanostane-treated breast cancer SK-BR-3 cells. The Figure shows that lanostane induces apoptosis in SK-BR-3 cells in a dose-dependent manner. The experiments were performed in triplicate.

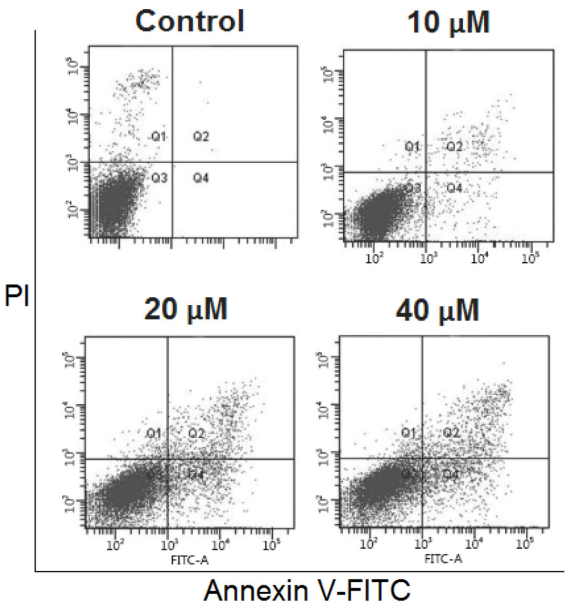

Figure 5. Annexin V/PI staining of the lanostane-treated breast cancer SK-BR-3 cells. The Figure shows that the percentage of the apoptotic SK-BR-3 cells increased with increase in the concentration of lanostane. The experiments were performed in triplicate.

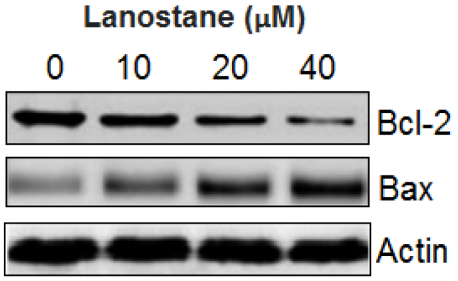

Figure 6. Western blotting showing the expression of Bax and Bcl-2 in lanostane-treated breast cancer SK-BR-3 cells. The Figure shows that the expression of Bax increased and that of Bcl-2 decreased with increasing concentrations of lanostane. The experiments were performed in triplicate.

els of active proteins, i.e., GTP-RhoA (p-RhoA) and phosphorylated ROCK II (p-ROCK II) decreased considerably (Figure 9). The inhibitory effects of lanostane on ROCK II signaling were concentration-dependent. The decline in p-RhoA and p-ROCK II protein levels were suggestive of inactivation of ROCK signaling which is necessary for cancer proliferation and particularly for its metastasis to bone.

Discussion

Although the incidence of cancer cases have declined in last two to three decades, current estimates have revealed an alarming truth that even in developed countries, human cancer will be the leading mortality cause in the near future [18]. Current understanding about cancer progression has revealed fascinating facts about the molecular mechanisms controlling cancer proliferation and metastasis. Of such mechanisms, Rho-associated kinase (ROCK) signaling has been revealed to play a prominent role in cancer development. The ROCKS with two isomers (ROCKI and ROCK II) are under the control of Rho-GTPases. Among these GTPases, RhoA is most studied and when bound to GTP, gets activated, phosphorylates the ROCKS and initiates ROCK signaling [19]. The ROCK signaling is important for regulating the morphology of cells, cell cycle, proliferation and progression of human cancers [20]. Breast cancer constitutes the most dominant malignancy in females with high mortality rates [21]. The deaths caused by breast cancer are mainly due to the metastasis of cancer cells to other tissues, mainly the lungs and bones. Studies have shown that the ROCK signaling is important for metastasis of breast cancer to bone [17]. Experiments carried out in *in vivo* systems revealed that the metastasis to bone was remarkably inhibited when ROCK signaling was inactivated by employing signaling inhibitors [16]. In the present study, we found that lanostane has a potential to inhibit the proliferation of breast cancer cells. The antiproliferative effects increased with increasing concentrations of the molecule. However, the growth inhibitory effects were significantly lower

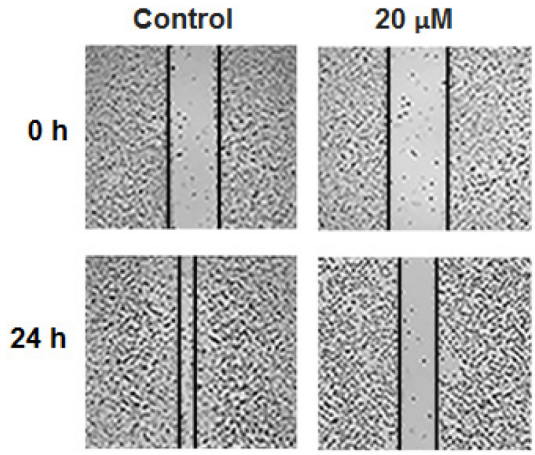

Figure 7. Wound healing assay of the lanostane-treated breast cancer SK-BR-3 cells. The Figure shows that lanostane inhibited considerably the migration of these cells. The experiments were performed in triplicate.

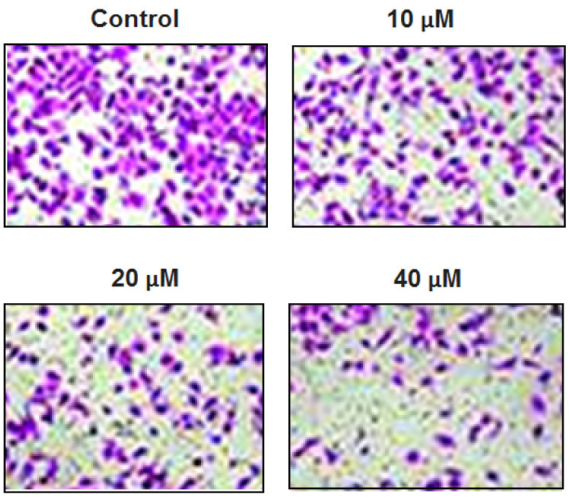

Figure 8. Transwell assay of the lanostane-treated breast cancer SK-BR-3 cells. The Figure shows that lanostane inhibited the invasion of these cells dose-dependently. The experiments were performed in triplicate.

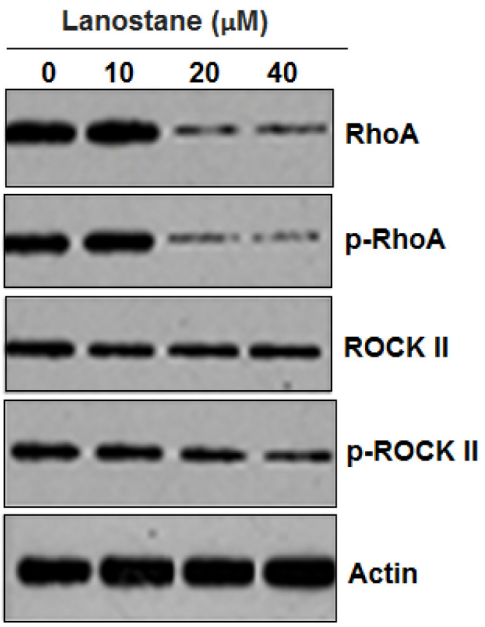

Figure 9. Western blot analysis of the lanostane-treated breast cancer SK-BR-3 cells showing that the molecule blocked the Rho-associated kinase signaling in a dose-dependent manner. The experiments were performed in triplicate.

when normal breast cells were treated with lanostane. Further, the molecule induced Bax/Bcl-2 signaling mediated apoptosis in cancer cells, the level of which increased with increasing dosage.

The effectiveness of natural products like melatonin in restricting the breast cancer growth by inducing cell apoptosis is already known [22]. Melatonin was further seen to inhibit the cancer cell metastasis by inhibiting the ROCK signaling. Our results are in coherence with these previous reports. Lanostane was seen to inhibit the migration and invasion of cancer cells, i.e the metastasis. When the inhibition of metastasis was modulated through ROCK signalling, the concentration of RhoA GTPase and ROCK II proteins was determined. The concentration of native proteins remained the same under lanostane treatment but there was a significant decline in GTP bound RhoA and phosphorylated ROCK II. This result suggests that lanostane is active in inhibiting the activation of RhoA by preventing the addition of GTP to it, the consequence of which is decline in the phosphorylated ROCK II, which renders ROCK signaling pathway inactive. As discussed above, ROCK signaling is necessary for metastasis of breast cancer to the bone and as such it is evident that lanostane inhibits bone metastasis by inhibiting the ROCK signaling.

Conclusion

From the results of current study it can be suggested that the understanding the molecular basis of disease development, specifically cancer and in particular breast cancer will help better administration of anticancer strategies by targeting the underlying molecular pathways.

Conflict of interests

The authors declare no conflict of interests.

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