JBUON 2020; 25(3): 1463-1468
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
Email: editorial_office@jbuon.com

ORIGINAL ARTICLE

Anticancer effects of Lanostane against human gastric cancer cells involves autophagy, apoptosis and modulation of m-TOR/PI3K/AKT signalling pathway

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Summary

Purpose: *Gastric carcinoma is the fourth leading cause of cancer-related morbidity throughout the globe. There are limited clinical therapies for gastric cancer due to lack of effective drugs and ambiguity in molecular mechanisms. As such there is a pressing need for novel and effective anticancer drugs for gastric cancer. The main aim of the current research work was to investigate the anticancer effects of Lanostane natural product in MKN-45 human gastric cancer cells along with evaluating its effects on cell autophagy, apoptosis, and m-TOR/PI3K/AKT signalling pathway.*

Methods: *MTT cell cytotoxicity assay was used to evaluate cell viability of MKN-45 human gastric cancer cells. Apoptosis was evaluated by fluorescence microscopy using Hoechst 33258 and Annexin-V/propidium iodide (PI) assay using flow cytometry. Autophagy was evaluated by transmission electron microscopy (TEM) and western blot method. Effects on m-TOR/PI3K/AKT related protein expression were evaluated by western blot method.*

Results: *Lanostane molecule led to substantial and dose-dependent growth inhibitory effects on MKN-45 human gastric cancer cells. Clonogenic assay showed significant decrease in MKN-45 cell colonies. Hoechst 33258 and annexin V/PI revealed that lanostane induced dominant apoptotic effects in these cells and exhibited dose-dependence. TEM revealed that lanostane induced autophagy in MKN-45 cells by forming autophagosomes and autophagic vacuoles. Lanostane also targeted m-TOR/PI3K/AKT signalling pathway by altering the expression of some key proteins.*

Conclusion: *Lanostane displayed strong anticancer effects in MKN-45 human gastric cancer cells by triggering apoptosis and autophagy and targeting m-TOR/PI3K/AKT signalling pathway.*

Key words: *gastric cancer, lanostane, fluorescence microscopy, apoptosis, autophagy*

Introduction

Gastric carcinoma (GC) is among highly lethal malignancies of the digestive tract, accounting for high number of deaths globally. In 2002, approximately 0.9 million new cases and 0.65 million deaths were recorded [1]. In addition to geographical variation GC presents higher incidence in develop-

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Received: 26/09/2019; Accepted: 18/10/2019

ing countries. Occurrence and development of GC are closely associated with telomerase and telomere. Telomerase sustains chromosomal integrity and telomere length during early stages of life in recurrently dividing cells. During adulthood telomerase remains dormant in somatic cells but reactivates in cancer cells and keeps the small length of telomeres in hastily multiplying cells leading to swift cancer cell progression [2,3]. Compared to other cancers, GC has a very poor prognosis and the only available complete treatment is surgical resection. Commonly used therapeutic agents like fluoropyrimidine and platinum-based drugs haven't proved successful in increasing overallal survival [4,5]. Thus lack of effective chemotherapeutic drugs with least or no side-effects remains a major hurdle in GC treatment [6]. To control and reduce the incidence of GC there is an urgent need for novel strategies and chemotherapeutic drugs with lower or no side-effects. Natural products and their derivatives (especially plant isolates) have served as a pool of pharmacologically important compounds which are used in the treatment of numerous diseases [7-11]. Naturally occurring triterpenes prevailing in animals, vegetables and fungi exhibit several medicinal implications including anticancer, antifungal, anti-inflammatory, anti-viral, anti-bacterial as well as anti-oxidative and many other properties [12-17]. Lanostane, a naturally occurring triterpene, has revealed various medicinal implications against various human diseases including glioma, leukemia, melanoma, colon cancer, pancreatic cancer, hepatic cancer, ovarian cancer, breast cancer, lung cancer as well as gastric cancer [18,19]. Lanostane has revealed induction of cytotoxicity in gastric A2521 carcinoma cell line. The current study was carried out to examine the anticancer potencial of Lanostane triterpene against human gastric cancer along with evaluating its effects on cellular autophagy, apoptosis and m-TOR/PI3K/AKT signalling pathway.

Methods

Assessment of cell cytotoxicity

The cytotoxic effect of Lanostane against human MKN-45 gastric cancer cell line was assessed through MTT cytotoxicity assay. MKN-45 cells were seeded at a density of 4×10^5 cells per well in 96-well plates. Seeded cells were incubated for attachment at 37°C for 24 h. Incubation was followed by treatment with varying doses of Lanostane i.e. control, 2.5, 5, 10, 20, 40 and 80 µM. Treated cells were further incubated for 24 and 48 h and consequently each well was added 10 µl of MTT solution (5mg/ml) and incubated for 5 h at 37°C. The formazan crystals thus formed were dissolved in 100µl of dimethyl sulfoxide (DMSO) by mild shaking. Afterwards, absorbance was measured at 490nm on microplate reader (Bio-Rad, USA) and the percentage of cell survival was compared against the untreated controls.

Clonogenic assay

MKN-45 GC cells were harvested at exponential growth phase and numbered with hemocytometer. 400 cells per well were seeded, followed by incubation till cells got attached. Attached cells were then treated with Lanostane with control, 10, 40 and 80 µM. Treated MKN-45 cells were then uninterruptedly incubated at 37°C for 96 h. Thereafter, phosphate-buffered saline (PBS) was used for washing Lanostane-treated cells and colony fixation was performed with methanol. Finally, colonies were stained with crystal violet and examined under light microscope.

Nuclear morphology detection via Hoechst 33258

Fluorescent nuclear dye Hoechst 33258 was used to analyze apoptosis induction by Lanostane in MKN-45 cells. In brief, at a concentration of 4×10^5 cells/well MKN-45 GC cells were seeded in 6-well plates and incubated for 12 h at 37°C. Seeded cells were then exposed to changing doses of Lanostane i.e. control, 10, 40 and 80 µM for 12 h. Washing of Lanostane-treated cells was done with PBS followed by incubation with 5 µg/ml Hoechst 33258 for 15 min at 37°C in humidified dark chamber. Inverted fluorescence microscope (Nikon ECLIPSE Ti-S, Japan) was used to capture images of the Lanostane-treated cells.

Analysing apoptosis via annexin V-FITC/PI double staining assay

The apoptotic effect of Lanostane against MKN-45 GC cells was analysed with annexin V-FITC/PI apoptosis kit (BD Biosciences, San Diego, CA, USA). Cancer MKN-45 cells were seeded in 6-well plates, followed by Lanostane treatment for 24 h at changing doses (control, 10, 40 and 80 µM). Treated cells were washed three times with PBS, resuspended in annexin V binding buffer (Biolegend®, USA)at 1×10^5 cells/ml and 100 µl of solution was drawn out and transferred to 6-well culture plates (5 ml). Thereafter, 5 µl of Annexin V-FITC conjugate and PI were supplemented to cell suspension followed by incubation at room temperature in the dark. Finally, 1×binding buffer was supplemented to cell suspension and immediately examined on FACS Calibur flow cytometer (BD Biosciences, San Diego, CA, USA).

Autophagy analysis via transmission electron microscopy (TME)

After treatment with 40 µM Lanostane, MKN-45 GC cells were fixed with 4% glutaraldehyde in 0.05M sodium cacodylate buffer. Osmium tetroxide 1.5% was used for post-fixation and dehydration of these cells was done with alcohol. Finally, MKN-45 treated cells were evenly organised for implantation in Epon 812 and then examined under Zeiss CEM 902 electron microscope.

Western blotting

Human MKN-45 GC cells were lysed with RIPA lysis buffer (Sigma Aldrich, USA) bearing protease in-

hibitor. Afterwards, using SDS-PAGE 7.5% protein extracts were separated followed by transference to PVDF membranes (polyvinylidene difluoride). Blocking the membranes was done with fat free milk for 1 h at room temperature. Primary antibody treatment overnight at 4°C was followed by incubation with secondary antibody. Finally, the membranes were visualized under enhanced chemiluminescence and quantification of protein signals was performed by scanning densitometry via bio-image analysis software (Bio-Rad, USA).

Statistics

Data are shown as mean±SD. ANOVA (one way analyses of variance): Scheffe's test for *post hoc* comparisons was used to evaluate significant intergroup differences along with considering $p<0.05$ as statistically significant.

Results

Lanostane induced cytotoxicity and inhibited colony formation in human MKN-45 GC cells

MTT cytotoxicity assay was performed to assess the impact of Lanostane treatment to human MKN-45 GC cells (Figure 1) and showed that the viability of MKN-45 cells decreased massively from 100 to near about 10% after increasing the Lanostane dose from 0l to 80 µM (Figure 2), thus indicating the dose-dependent induction of cytotoxicity by Lanostane in MKN-45 GC cells. Lanostane was also tested for its effect on colony formation and the results revealed that after Lanostane exposure (at varying doses of control, 10, 40 and 80 µM) the colony size and number started to decrease first and ultimately started to vanish at higher doses (Figure 3). Thus MTT and clonogenic assay showed that Lanostane is a potential cytotoxicity inducer and cancer cell colony inhibitor.

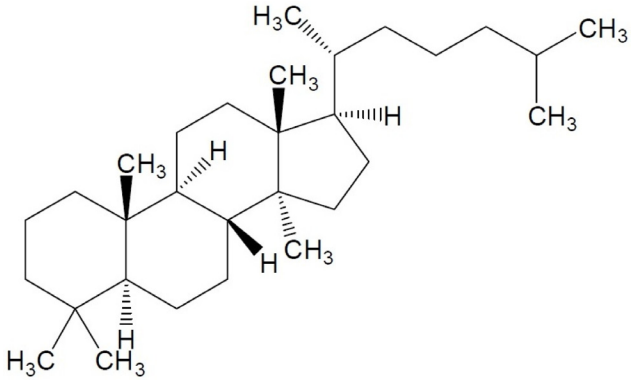

Figure 1. Chemical structure of Lanostane triterpene.

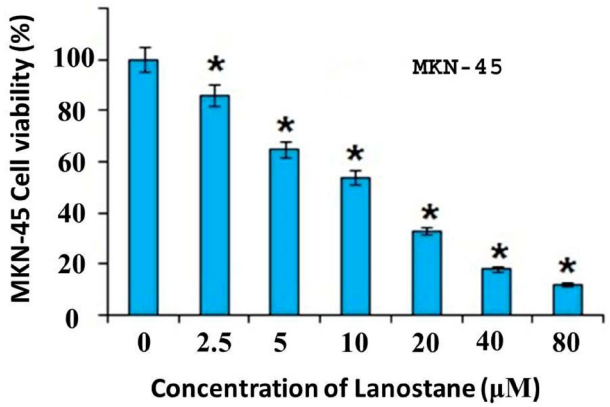

Figure 2. MTT cell viability assay for determination of effects of Lanostane on cell proliferation against human MKN-45 GC cells. The experiments were repeated three times and data are expressed as mean±SD (*$p<0.05$).

Analysis of apoptosis induction by Lanostane in human MKN-45 GC cells

Further investigations were carried out to validate whether the cytotoxic effects of Lanostane were apoptosis-driven. Thus Hoechst 33258 staining and Annexin V-FITC/ PI dual staining assays were performed for apoptosis analysis. The results from Hoechst 33258 staining confirmed that after treatment with different Lanostane doses i.e. control, 10, 40 and 80 µM, cell morphology showed significant changes including fragmented, dislocated, shrunken and condensed nucleus with greater fluorescence along with randomly distributed chromatin in contrast to the controls which revealed no significant changes with lower fluorescence. Thus results from Hoechst 33258 indicated nuclear damage in treated cells as compared to

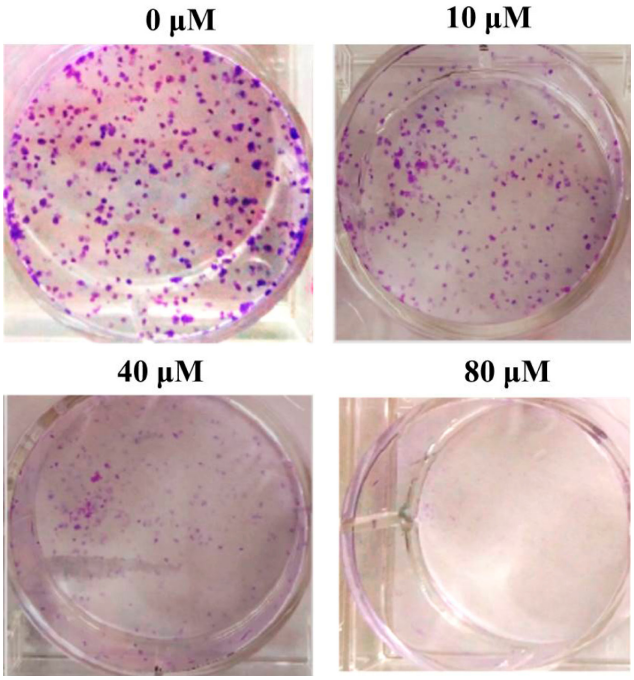

Figure 3. Impact of Lanostane treatment at indicated doses on colony formation of human MKN-45 GC cells. The experiments were repeated three times.

controls, confirming apoptotic cell death (Figure 4). Further, quantifying the rate of apoptosis via annexin V-FITC/PI dual staining assay confirmed that on increasing Lanostane concentration the number of apoptotic cells increased significantly (Figure 5), thus indicating that the cytotoxicity induction by Lanostane is apoptosis-mediated.

Induction of autophagic cell death in human MKN-45 GC cells by Lanostane

Further, transmission electron microscopy (TEM) was performed to confirm whether the cytotoxic effects were governed by autophagy as well and observed the cell morphology after Lanostane treatment (at control, 10, 40 and 80 μM). Images were captured at different doses revealing formation of autophagic vesicles (Figure 6). Autophagic vesicles indicated onset of autophagy and hence we also concluded that not only apoptosis but autophagy was also responsible for cytotoxicity of Lanostane molecule. Western blotting analysis was also performed to check the levels of autophagy-associated proteins and it too supported the fact that cytotoxicity of Lanostane is due to autophagy induction. The levels of LC3B-I, LC3B-II and Beclin-1 all increased in a dose-dependent manner (Figure 7).

Lanostane modulated the m-TOR/PI3K/AKT signalling pathway

Western blotting analysis was performed to check the levels of m-TOR/PI3K/AKT signalling pathway related proteins. Treatment with changing concentrations of Lanostane showed that the

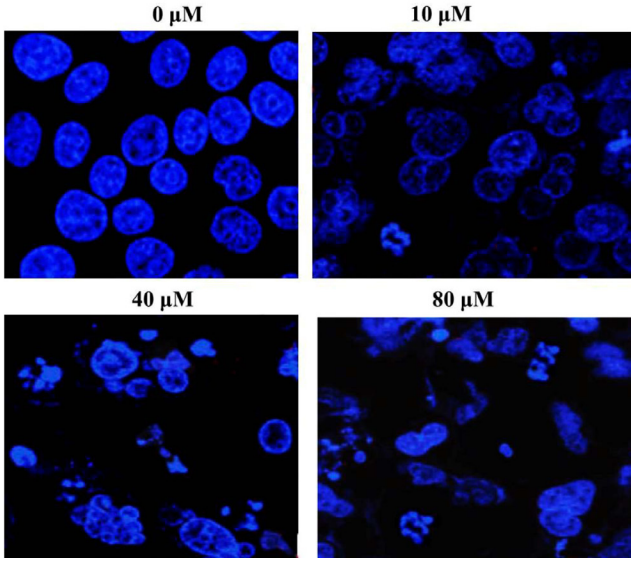

Figure 4. Hoechst 33258 staining for morphology determination after Lanostane treatment of human MKN-45 GC cells. The experiments were repeated three times.

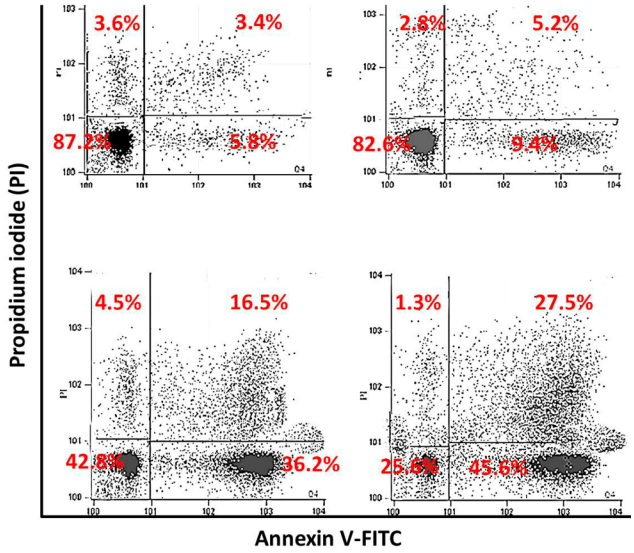

Figure 5. Annexin V-FITC/PI dual staining assay for quantification of apoptosis induction by Lanostane in human MKN-45 GC cells. The experiments were repeated three times and data are expressed as means±SD.

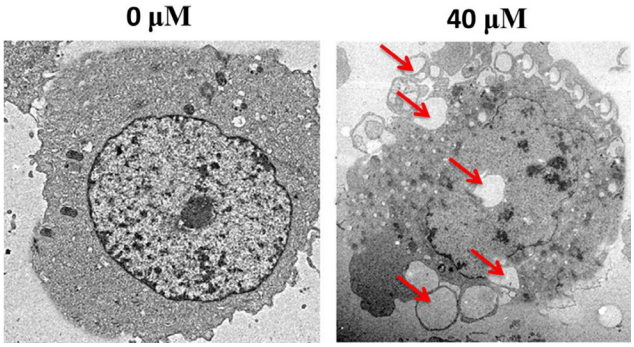

Figure 6. Autophagy analysis via transmission electron microscopy at indicated doses. The experiments were repeated three times. The red arrows indicate autophagic vesicles which proves that Lanostane induces autophagy.

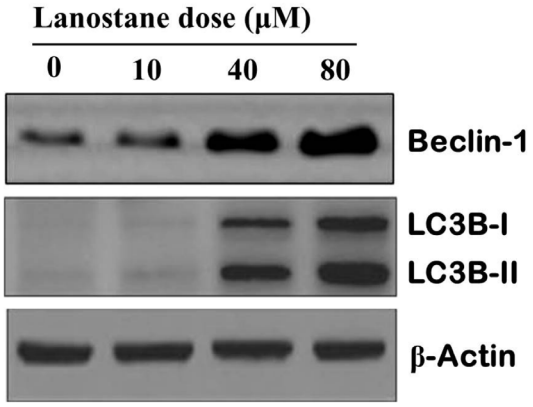

Figure 7. Western blotting analysis for evaluation of the impact of Lanostane on the levels of autophagy-related proteins like LC3B-I, LC3B-II and Beclin-I. The experiments were repeated three times.

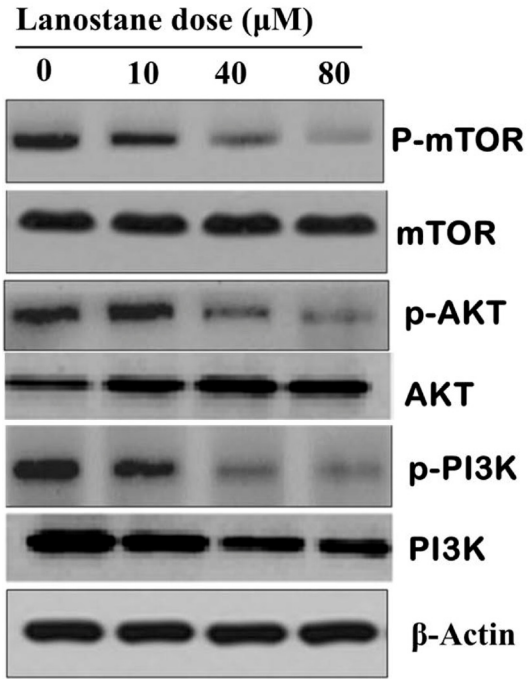

Figure 8. Western blotting analysis revealing the effects of Lanostane molecule treatment on m-TOR/PI3K/AKT signalling pathway of human MKN-45 GC cells. The experiments were repeated three times.

expressions of p-mTOR, p-AKT, p-PI3K and PI3K decreased remarkably and AKT levels increased. In addition, it was observed that the expression of mTOR remained constant. Thus we may conclude that Lanostane interfers with m-TOR/PI3K/AKT signalling pathway and targets it to induce cytotoxicity in human MKN-45 GC cells (Figure 8).

Discussion

In the recent past GC treatment has gone through productive advancements and developments in enhancing the rate of overall survival [20]. Still, GC has higher prevalence like in East Asian countries where half of the cancer cases are GC [21]. There are several molecular pathways which can be targeted for cancer treatment including apoptosis as well as autophagy. Apoptosis and autophagy are both natural phenomena of programmed cell death. Apoptosis is characterized by formation of apoptotic crops and autophagy by formation of autophagosomes or autophagic vesicles. Herein Lanostane triterpene was investigated for its anticancer potency against human MKN-45 GC cells. Cytotoxicity induction in human MKN-45 GC cells by Lanostane was estimated via MTT cell proliferation assay, revealing remarkable cytotoxicity induced by this molecule, suggesting its potent anti-proliferative potential. Further, the clonogenic assay was performed to examine the effects of Lanostane on colony formation ability of MKN-45 GC cells. The results depicted significant dose-related suppression of colony formation. Also, the cytotoxic effects of Lanostane were examined to see whether they are mediated via apoptosis or autophagy or both. Apoptosis analysis was performed via Hoechst 33258 staining and Annexin V-FITC/PI dual staining, revealing noteworthy changes in nuclear morphology like shrunken, condensed and fragmented nucleus as well as dispersed chromatin indicating apoptosis. Quantification of apoptosis with Annexin V-FITC/PI assay revealed tremendous increase in the percentage number of apoptotic cells as the Lanostane dose increased, thus clearly indicating that cytotoxicity of the current tested molecule was due to apoptosis induction. Next, autophagy-related studies were carried out through TEM as well as western blotting revealing important observations like formation of apoptotic vesicles/autophagosomes and increased levels of LC3B-I, LC3B-II and Beclin-1 in a dose-dependent manner which indicate autophagy. Hence it is clear that cytotoxicity of Lanostane is due to apoptosis as well as to autophagy induction. Finally, the impact of Lanostane exposure on m-TOR/PI3K/AKT signalling pathway of MKN-45 cancer cells was assessed through western blot assay, unveiling significant increase in AKT and decline in the levels of p-mTOR, p-AKT, p-PI3K and PI3K with constant mTOR levels, indicating blockage of m-TOR/PI3K/AKT signalling pathway.

Conclusion

All the above assays, observations and discussions revealed remarkable anticancer potential of Lanostane against human MKN-45 GC cells through induction of apoptosis and autophagy. Lanostane also blocked m-TOR/PI3K/AKT signalling pathway indicating its potency as a lead molecule in GC treatment.

Funding support

This study was supported by Hubei Municipal Health and Family Planning Commission (No: WJ2017Q034), and Wuhan Municipal Health and Family Planning Commission (No:WX16E11).

Conflict of interests

The authors declare no conflict of interests.

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