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Original Research Article

Gastric cancer cell proliferation is inhibited by α-santonin via targeting of PI3K and AKT activation

Lin Nie, Lijiu Zhang*
Department of Gastroenterology, The Second Hospital of Anhui Medical University, Hefei City, Anhui Province 230601, China

For correspondence:* ***Email:* *xiyangone@sina.com;* ***Tel:*** *+8613956976104*

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Abstract

Purpose: *To investigate the effect of α-santonin on proliferation of gastric cancer cells.*
Methods: *Cell proliferation was analysed by 3-4-5-Dimethylthiazol-2-yl-25-diphenyltetrazolium bromide (MTT) assay and migration by wound healing assay. Matrigel coated Transwell chamber was used for determination of cell invasion. Expression of proteins and mRNA was assessed using western blot and RT-PCR assay, respectively.*
Results: *In NUGC4 and MKN45 cell cultures, treatment with α-santonin promoted miR 145 expression significantly when compared to control. Treatment of NUGC4 cells with α-santonin for 48 h significantly increased apoptosis in comparison to control. At 100, 150 and 200 μM concentrations of α-santonin, the level of cell apoptosis increased to 45, 53 and 64 %, respectively ($p < 0.05$). Treatment with α-santonin caused NUGC4 cell population increase in G1/G0 phase with reduction in S and G2/M phases. A significant reduction in NUGC4 cell invasion was observed following treatment with α-santonin. The α-santonin treatment of NUGC4 cells at 200 μM concentration markedly reduced cell invasion ($p < 0.05$). Treatment of NUGC4 cells with α-santonin reduced the expression of c Myc, PI3K, and p AKT. The production of MMP-2 and MMP-9 in NUGC4 cells was also decreased by α-santonin treatment.*
Conclusion: *The study demonstrates that α-santonin plays important role in inhibition of gastric cancer cell proliferation by arrest of cell cycle and apoptosis induction. Moreover, the activation of PI3K and AKT was also suppressed by α-santonin. Therefore, α-santonin can potentially be used for the treatment of gastric cancer.*

Keywords: *Apoptosis, MicroRNA, Tumor suppressor, Metastasis, Infiltration*

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INTRODUCTION

Gastric cancer, the second most common cause of deaths associated with cancer, ranks fourth among different types of cancers detected worldwide [1]. The number of gastric cancer cases detected is increasing every year [2]. In the East Asian region the incidence rate of gastric cancer is very high and prognosis rate is very low [2]. In most of the cases gastric cancer is detected at an advanced [3]. Failure to detect gastric cancer at an early stage is one of the main reasons responsible for poor prognosis of the patients [4]. Gastric cancer cells possess the potential to undergo metastasis and infiltration at a very high rate which is the major hindrance to

available treatment strategies [5]. The discovery of molecules which can inhibit metastasis and infiltration of gastric cancer cells as well as suppress their proliferation can form an effective treatment strategy.

MicroRNA (miRNA), generally comprised of 19-25 nucleotides control the translation inhibiting genes through regulation of one or more mRNAs [6]. The miRNAs play important role in various cellular processes like proliferation, cell death and development of the organs [7]. Studies have shown that miRNAs act as key factors in gastric carcinoma development and progression [8]. The miR-145 acts as a tumor-suppressor miRNA by regulating tumor cell growth through targeting the expression of proto-oncogene protein (c-Myc) and POU domain [9]. It is reported that miR-145 plays inhibitory role in gastric cancer by inhibiting proliferation and tumor metastasis through suppression of MYO6 [10]. Studies investigating mechanism of gastric cancer inhibition by miR-145 have found down-regulation of Sp1 and N-cadherin protein translation [11]. Thus, it is believed that miR-145 suppresses gastric cancer by targeting various factors.

A sesquiterpene lactone, *α*-santonin, isolated from the plant, *Artemisia santonica,* was initially used as anthelminthic molecule [12]. At present *α*-santonin is used as an important substrate for the synthesis of eudesmanolide compounds [12]. Studies have shown that *α*-santonin possesses several biological properties like cytotoxic, antioxidant and anti-inflammatory [12,13]. The synthetic derivatives of *α*-santonin were found to be more potent as anti-cancer [13] and immunosuppressant [13] compounds than the parent molecules. In the present study effect of α-santonin on proliferation and metastasis of gastric cancer cells was investigated and also the mechanism involved was studied. The study has demonstrated that α-santonin exhibits inhibitory effect on gastric cancer cell proliferation by arrest of cell cycle and apoptosis induction. The activation of PI3K and AKT was also suppressed by α-santonin.

EXPERIMENTAL

Cell culture

The NUGC4 and MKN45 gastric carcinoma cell lines were supplied by Cell Bank, Chinese Academy of Sciences (Shanghai, China). The cells were cultured in RPMI-1640 medium (Sigma) mixed with FBS (10 %) and antibiotics (1 %). The conditions used for culture in the incubator were 37˚C in a 5 % CO_2 humid atmosphere.

Evaluation of cell proliferation

The 3-4-5-dimethylthiazol-2-yl-25-diphenyltetrazolium bromide colorimetric assay was used for the assessment of effect of different concentrations of α-santonin on NUGC4 and MKN45 cell proliferation. The cells plated in 96-well plates at 1 x 10^6 cells per well density were treated for 48 h with 20, 40, 60, 80, 100, 150 and 200 μM concentrations of α-santonin in DMEM. Then 20 μL MTT solutions (5 mg/mL) was added into each of the well and cell incubation was continued for 5 h more. The medium was removed and dimethyl sulfoxide (150 μL) was added to the plates for dissolving any insoluble material formed. The plates were kept in shakers for 10 min before recording optical density using multi-well spectrophotometer at a wavelength of 482 nm.

Apoptosis analysis

Apoptosis analysis in NUGC4 cell cultures on treatment with 100, 150 and 200 μM concentrations of α-santonin or DMSO (control) was assessed by annexin V/PI kit in accordance with the manufacturer's protocol. Briefly, after 48 h of treatment with α-santonin, the cell pellets were put in to the 1x binding buffer. The cells were then incubated with Annexin V (5 ml) and PI (10 mL) for 10 min under complete darkness. The flow cytometer (Epics-XLII, Becman Coulter, Inc., Brea, CA, USA) was employed for the measurement of cell fluorescence.

Cell cycle analysis

Cell cycle distribution in NUGC4 cells after treatment with 100, 150 and 200 μM concentrations of α-santonin or DMSO (control) was examined by flow cytometry. Briefly, following 48 h treatment in 6-well plates with α-santonin the cells were harvested and subsequently fixed in 70% ethyl alcohol overnight at 4˚C. Tris-hydrochloric acid buffer (pH 7.6) mixed with 1% RNase A was added to the cells and then stained with 5 mg/ml solution of propidium iodide. Flow cytometry was used for determination of DNA content distribution in the cells.

Cell invasion assay

Invasion potential of NUGC4 cells following treatment with α-santonin was examined in 24-well Transwell plates. The chamber inserts after coating with 200 mg/mL Matrigel were

subjected to overnight drying under sterile atmosphere. NUGC4 cells were treated with 100, 150 and 200 µM concentrations of α-santonin for 48 h and then put on the top chamber at 2 x 10^5 cell/mL density in RPMI-1640 medium mixed with 20% FBS. At 48 h, the upper chamber was cleaned off using cotton to remove the non-adhesive cells. The cells were fixed with 100% methyl alcohol for 20 min at room temperature. The fixed cells were stained with hematoxylin-eosin for 25 min at room temperature. The light microscope (Olympus Corporation, Tokyo, Japan) was used for calculation of cells invaded to the lower chamber in five random fields.

Cell migration assay

The effect of α-santonin on migration potential of NUGC4 cells was analysed by wound healing assay. In brief, the cells treated with 100, 150 and 200 µM concentrations of α-santonin for 48 h were seeded at 1.5 x 10^4cells/well density in 6-well plates. The monolayer of cells formed was scratched through the middle using the tip of a 200 µL pipette and non-adhesive cells were cleaned off. Migration of the cells through the wounded region was examined by inverted microscope and quantification was performed by Image-Pro Plus software version 7.0 (Media Cybernetics, Inc., Rockville, MD, USA).

Western blot analysis

NUGC4 cells were seed into 6-cm dishes at 1.5 x 10^6 cells/dish and incubated with α-santonin for 48 h. The cells were PBS washed thrice prior to total protein content extraction using 40 mM Tris-hydrochloric acid (pH 7.4) mixed with 150 mM sodium chloride and 1% Triton X-100 containing protease inhibitors. The quantification of proteins was by BCA protein assay kit and resolved with by 10-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were transferred to a PVD membrane which was blocked with skimmed milk (5%) and Tween-20 for 3 h at room temperature. The membranes were incubated with antibodies against c-Myc (dilution 1:1,000; cat. no. 5605), p-AKT (dilution 1:1,000; cat. no. 4060), PI3K (dilution 1:1,000; cat. no. 4249), MMP-2 (dilution 1:1,000; cat. no. 40994), MMP-9 (dilution 1:1,000; cat. no. 13667) and GAPDH (dilution 1:1,000; cat. no. 8884) all obtained from Cell Signaling Technology, Inc. The blots were twice washed with 1X PBST and then incubated for 2 h with horseradish peroxidase-conjugated secondary antibody at room temperature. The SignalFire™ Plus ECL Reagent was used for the visualization of immunoreactive bands. The quantification of bands was done using Image J version 2.0 software (Bio-Rad Laboratories Inc, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Isolation of total RNA from NUGC4 cells after 48 h of α-santonin treatment was made using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) in accordance with the manufacturer's protocol. cDNA synthesis was performed by ThermoScript RT-PCR system (Invitrogen; Thermo Fisher Scientific, Inc.) in accordance with the manufacturer's instructions. qPCR was carried out on cDNA using SYBR® Premix Ex Taq™ II kit (Takara Bio, Inc., Otsu, Japan). The reaction sequence consisted of 38 cycles: denaturation for 15 s at 93 ˚C, annealing for 25 s at 58 ˚C, and extension 45 s at 70˚C.

Gene	Forward	Reverse
MiR-145	5'-GTC CAG TTT TCC CAG GAA TCC CT-3'	5'-GCT GTC AAC GAT ACG CTA CCT A-3
U6	5'GCT TCG GCA GCA CAT ATA CTA AAA T3'	5'CGC TTC ACG AAT TTG CGT GTC AT3'

Statistical analysis

The data was analysed using SPSS version 17.0 software (SPSS, Inc, Chicago, IL, USA). Presented data are the mean ± standard deviation of triplicate experiments carried out independently. Analysis of the data was done by one-way analysis of variance followed by Tukey's post-hoc test. $p<0.05$ was taken as statistically significant difference.

RESULTS

α-Santonin inhibits NUGC4 and MKN45 cell proliferation

MTT assay results showed that NUGC4 and MKN45 cell proliferation were inhibited in a concentration-based manner by α-santonin (Figure 1). Effect of α-santonin on NUGC4 and MKN45 cell proliferation was assessed at 20, 40, 60, 80, 100, 150 and 200 µM concentrations. Treatment with 20, 40, 60, 80, 100, 150 and 200 µM concentrations of α-santonin reduced NUGC4 cell proliferation to 95, 82, 69, 55, 46, 39 and 32%, respectively. MKN45 cell proliferation was reduced to 93, 86, 72, 62, 54, 43 and 37%, respectively on treatment with 20, 40, 60, 80, 100, 150 and 200 µM concentrations of α-santonin. These findings proved that α-santonin suppressed NUGC4 and MKN45 cell proliferation.

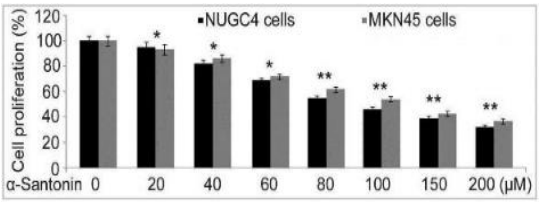

Figure 1: Effect of α-santonin on NUGC4 and MKN45 cell proliferation. α-santonin at different concentrations was added to NUGC4 and MKN45 cell cultures and proliferation was assessed by MTT assay; *p < 0.005 and **p < 0.001 vs. control cells

α-Santonin promotes miR-145 expression in NUGC4 and MKN45 cells

In NUGC4 and MKN45 cell cultures, treatment with α-santonin promoted miR-145 expression significantly in comparison to the control (Figure 2). The expression of miR-145 protein was also higher in α-santonin treated NUGC4 and MKN45 cells in comparison to the untreated cells. The expression of miR-145 protein and mRNA was in a concentration based manner when NUGC4 and MKN45 were treated with α santonin.

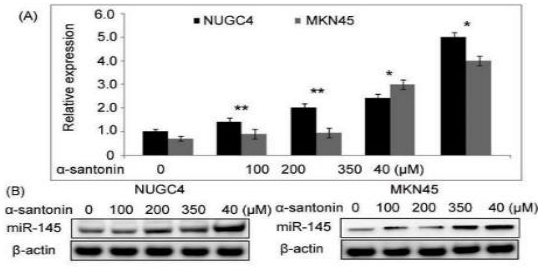

Figure 2: Effect of α-santonin on miR-145 in NUGC4 and MKN45 cells. (A) Western blot analysis of miR-145 protein expression and (B) RT-PCR assay for miR-145 mRNA expression in NUGC4 and MKN45 cells; *p<0.005 and **p < 0.001 vs. control cells

α-Santonin promotes NUGC4 cell apoptosis

Treatment of NUGC4 cells with α-santonin for 48 h significantly increased apoptosis in comparison to the control (Figure 3). The percentage of early as well as late apoptotic cells increased in concentration-based manner in NUGC4 cell cultures by α-santonin treatment. At 100, 150 and 200 μM concentrations of α-santonin apoptotic cell percentage increased to 45, 53 and 64 %, respectively, compared to 2.8 % in control cultures.

α-Santonin causes cell-cycle arrest in NUGC4 cells

NUGC4 cells treated with different (100, 150 and 200 μM) concentrations of α-santonin for 48 h were examined for cell cycle distribution (Figure 4). Treatment with α-santonin caused NUGC4 cell population increase in G1/G0 phase with reduction in S and G2/M phases.

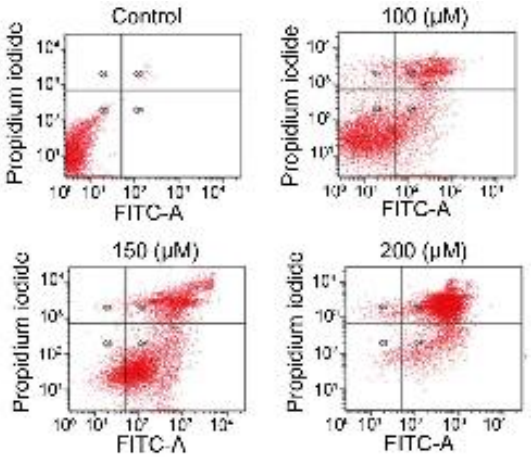

Figure 3: Effect of α-santonin on apoptosis in NUGC4 cells. The cells were treated with different concentrations of α-santonin for 48 and flow cytometry was used to measure apoptosis

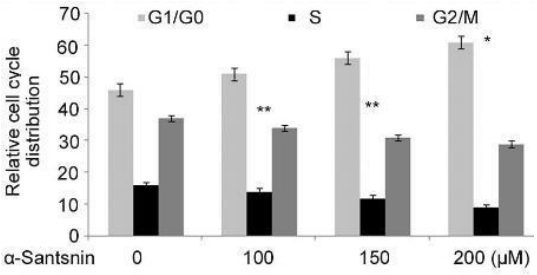

Figure 4: Effect of α-santonin on NUGC4 cell cycle distribution. α-santonin at different concentrations was added to NUGC4 cell cultures followed byflow cytometry; *p < 0.005 and **p < 0.002 vs. control cells

α-Santonin inhibits NUGC4 cell invasion and migration

The NUGC4 cells treated with 100, 150 and 200 μM concentrations of α-santonin for 48 h were examined by Matrigel Transwell assay (Figure 5 A). A significant reduction in NUGC4 cell invasion was observed on treatment with α-santonin in concentration-based manner. Theα-santonin treatment of NUGC4 cells at 200 μM concentration markedly reduced cell invasion in comparison to the control. The migration potential of NUGC4 cells was also suppressed significantly by α-santonin treatment in wound healing assay (Figure 5 B). The inhibitory effect of α-santonin on NUGC4 cell migration was maximum at 200 μM concentration.

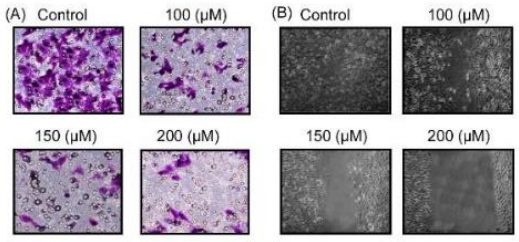

Figure 5: Effect of α-santoninon NUGC4 cell metastasis. (A) Determination of NUGC4 cell invasion by Transwell assay (B) and migration by wound healing method. NUGC4 cells were treated with different concentrations α-santonin for 48 h. Images taken at magnification, x200

α-Santonin down-regulates PI3K/AKT expression in NUGC4 cells

Treatment of NUGC4 cells with 80, 100, 150 and 200 μM concentrations of α-santonin reduced the expression of c-Myc, PI3K, and p-AKT (Figure 6). The production of MMP-2 and MMP-9 was also decreased by α-santonin treatment in NUGC4 cells. However, the expression of cell cycle protein, p21 was increased significantly in NUGC4 cells on treatment with α-santonin at 48 h.

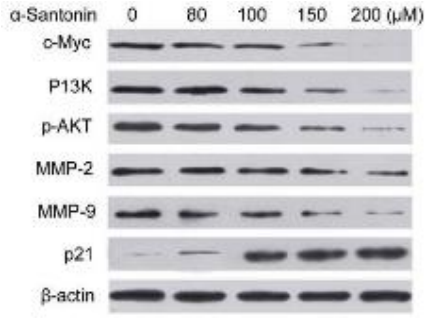

Figure 6: Effect of α-santoninon PI3K/AKT activation in NUGC4 cells. In NUGC4 cells treatment with different concentrations of α-santonin was followed by western blot assay to determine PI3K/AKT activation

DISCUSSION

The present study was designed to investigate the effect of α-santonin on gastric cancer cell proliferation and miRNA expression. The study showed that α-santonin treatment of gastric cancer cells suppressed proliferation and promoted miRNA expression. Treatment of gastric cancer cells with α-santonin led to arrest of cell cycle, activation of apoptosis and down-regulation of PI3K/AKT activation.

Gastric carcinoma one among the commonly detected malignancies accounts for more than a million new cases diagnosed globally every year [14]. Studies have shown that miR-145 expression is suppressed markedly in several types of carcinoma cells [15,16]. Down-regulation of miR-145 expression is believed to play a vital role in the development and proliferation of cancer [15,16].

In the present study the effect of α-santonin on proliferation of NUGC4 and MKN45 cells was analysed. The results showed that α-santonin treatment suppressed NUGC4 and MKN45 cell proliferation in a concentration-based manner. To investigate any involvement of miR-145 in α-santonin mediated suppression of NUGC4 and MKN45 cell proliferation, RT-PCR and western blot were used. The study showed that miR-145 expression was up-regulated by α-santonin treatment markedly in comparison to the control cultures. These findings suggested that α-santonin suppressed NUGC4 and MKN45 cell proliferation by up-regulation of miR-145 expression. It is reported that miR-145 acts as a tumor suppressor gene and its expression is markedly down-regulated during several cancers [17].

In the present study role of miR-145 up-regulation by α-santonin in various cellular processes was also studied. The results revealed that α-santonin treatment caused cell cycle arrest in NUGC4 cells in G1/G0 phase. In NUGC4 cells treatment with α-santonin markedly enhanced cell percentage in G1/G0 phase with reduction in S and G2/M phases. The rate of apoptosis in α-santonin treated NUGC4 cell cultures was also increased markedly than those of control cells. These results proved that α-santonin inhibits NUGC4 and MKN45 cell proliferation by activation of apoptosis and arrest of cell cycle through up-regulation of miR-145 expression.

The oncogene, c-Myc is associated with the proliferation, regulation of death and transformation of cancer cells [18]. Overexpression of c-Myc damages normal epithelial cells by inducing chromatin condensation [19]. In carcinoma cells arrest of cell cycle is promoted by suppression of c-Myc expression [19]. The proliferation of cancer cells is inhibited by targeting the expression of c-Myc [19]. In the present study α-santonin treatment of NUGC4 cells led to a significant reduction of c-Myc expression. Therefore, α-santonin induced miR-145 up-regulation and c-Myc down-regulation are involved in the inhibition of NUGC4 cell proliferation. MMP-2 and MMP-9 have well established role in the degradation of type IV collagen and thereby promote tumor development and metastasis [20].

The results from present study showed that α-santonin treatment inhibited levels of MMP-2 and MMP-9 in NUGC4 cells markedly in comparison to the control. The metastasis of NUGC4 cells was also inhibited on treatment with α-santonin in a concentration dependent manner. AKT activation plays a prominent role in the angiogenesis and metastasis of carcinoma cells by down-regulating the phosphorylation cascade [21]. In the present study α-santonin treatment prevented phosphorylation of AKT in NUGC4 cells.

CONCLUSION

The findings of this study show that α-santonin inhibits gastric cancer cell proliferation via up-regulation of miR-145 and down-regulation of c-Myc oncogene. Moreover, α-santonin treatment causes apoptosis activation and cell cycle arrest in NUGC4 cells. Therefore, α-santonin has a potential for development as a treatment strategy.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Lin Nie and Lijiu Zhangperformed the experimental work, carried out the literature study and compiled the data. Lijiu Zhang designed the study and wrote the paper. Both the authors thoroughly studied the paper before communication.

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