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Research Article

Z-Guggulsterone Inhibits Tumor Cell Proliferation, Migration *in vitro* and Tumor Growth *in vivo* through PTEN/Akt/COX-2 Signaling Pathway

Xiaopu He[1], Mengxue Sun[2], Peiyun Huang[1], Shuaishuai Zhuang[1], Weihao Sun[1*], Jie Hua[3*]

[1]Department of Geriatric Gastroenterology, Nanjing Medical University First Affiliated Hospital, Nanjing 210000, China;[2]Department of Geriatric, Huai'an Medical District of General Hospital of Eastern Theater Command, Huai'an 223000, China;[3]Department of Gastroenterology, Nanjing Medical University First Affiliated Hospital, Nanjing 210000, China

ABSTRACT

Cyclooxygenase-2 (COX-2) plays an important role in the carcinogenesis and progression of gastric cancer. Z-guggulsterone has been used as an antibacterial and anticancer agent; however, effects and action mechanism of Z-guggulsterone on the human gastric cancer cells remain unclear. This study evaluated the antitumor effects of Z-guggulsterone on human gastric cancer both *in vitro* and *in vivo* . Our results showed that Z-guggulsterone significantly inhibited cellular proliferation, migration and induced apoptosis *in vitro* , as well as inhibited tumor growth *in vivo* . In addition, Z-guggulsterone significantly inhibited the expression of Bcl-2, p-Akt and COX-2 as well as increased Bax and PTEN expression in gastric cancer cells. The PTEN inhibitor reversed Z-guggulsterone -induced down regulation of p-Akt and COX-2 expression. The Akt inhibitor combined with Z-guggulsterone can down-regulate the expression levels of p-Akt and COX-2, and the combined effect was better than that of Z-guggulsterone alone. These results collectively indicate that Z-guggulsterone inhibits proliferation and induces apoptosis of gastric cancer cells may be mediated by down-regulation of COX-2 expression through PTEN/Akt signaling pathway.

Keywords: Z-guggulsterone ; Gastric cancer; Cyclooxygenase-2; Proliferation; Apoptosis; PTEN

ABBREVIATIONS

BMI: Body Mass Index; ESI-MS: Electron Spray Ionization Mass Spectroscopy; Fet A: Fetuin A; pFet A: phosphorylated Fetuin A; MALDI-TOF-MS: Matrix Assisted Laser Desorption Ionization; OGTT: Oral Glucose Tolerance Test; rh: Human Recombinant; Ser: Serine; SFA: Saturated Free Fatty Acids; MUFA: Monounsaturated Free Fatty Acids; PUFA: Polyunsaturated Free Fatty Acids; Thr: Threonine; uSFA: Sum of Unsaturated Free Fatty Acids.

INTRODUCTION

Gastric cancer is the fourth most prevalent cancer and the second most common cause of cancer-related deaths worldwide [1-3]. Although overall survival in patients with gastric cancer has greatly improved in recent years due to increase detection of early cancers and wider implementation of radical surgery [4,5], the prognosis of advanced cancer remains poor as safe and effective adjuvant therapy options are limited [1]. Chemotherapy is the standard of care for incurable advanced gastric cancer. Our previous studies have shown that traditional natural drugs possessed significant anti-tumor potential for gastric cancer therapy [6-9].

Cyclooxygenase-2 (COX-2), the mitogen-inducible isoform, is constitutively expressed in gastric cancer and is related to cell proliferation and apoptosis, tumor invasiveness and metastasis [10-12]. Previous studies have shown that COX-2 inhibition by selective COX-2 inhibitors or small interfering RNA (siRNA) suppresses cell proliferation and induces apoptosis in human gastric cancer cells [9,13-15]. PTEN (gene of phosphate and tension homology deleted on chromosome ten) is a tumor suppressor gene with phosphatase activity that can regulate cell survival, proliferation, and energy metabolism processes [16,17]. Recent studies have demonstrated that the PTEN/Akt pathway can regulate COX-2 expression in human cervical cancer, gall bladder cancer and colorectal cancer [18-20].

Guggulsterone [4,17,20-pregnadiene 3,16-dione], a plant polyphenol obtained from the gum resin of the Commiphora mukul tree, has been used as an effective herbal medicine in the treatment of various diseases including cancers [21-23]. Guggulsterone exists as two isoforms: E- and Z-guggulsterone, shown in Figure 1 [24]. Recent studies have shown that Z-guggulsterone can induce apoptosis in liver, breast, and prostate cancer cells [25-27]. However, effects and exact molecular mechanism of Z-guggulsterone on human gastric cancer remain unclear. In the present study, human poorly differentiated MGC803, BGC823 and moderately differentiated

Correspondence to: Hua J, Department of Gastroenterology, Nanjing Medical University First Affiliated Hospital, Nanjing 210000, China, E-mail: huajie@njmu.edu.cn

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SGC7901 gastric cancer cell, in which COX-2 was found to be expressed [8,9,11,28,29], were applied to investigate the antitumor effects of Z-guggulsterone *in vitro* and *in vivo*. In addition, the expression of COX-2, PTEN, Akt and apoptosis-related proteins were detected to further elucidate the possible mechanism underlying the anti-tumor effects of Z-guggulsterone against gastric cancer.

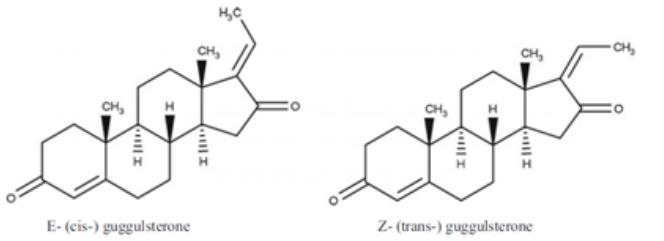

Figure 1: The molecular structure of E-guggulsterone and Z-guggulsterone.

MATERIALS AND METHODS

Reagents

Z-guggulsterone (MF: C21H28O2, MW: 312.45, CAS: 39025-23-5, purity: >98%), Dimethylsulfoxide (DMSO) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylformazan (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Z-guggulsterone was dissolved in DMSO as a 10 mg/ml stock solution and stored at -20°C. PTEN inhibitor bpv (HOpic) and Akt inhibitor MK2206 were purchased from Selleck Chemicals (Houston, TX, USA). Hoechst 33342 were purchased from Beyotime Institute of Biotechnology (Nantong, China). RPMI-1640 medium, Fetal Bovine Serum (FBS) and penicillin/streptomycin were purchased from Gibco BRL (Grand Island, NY, USA). Other reagents were of analytic grade and obtained from Nanjing Chemical Reagent Co. (Nanjing, China), unless otherwise described.

Cell lines and culture conditions

Human poorly differentiated MGC803, BGC823 and moderately differentiated SGC7901 gastric cancer cell lines were obtained from Shanghai Institute of Cell Biology (Shanghai, China). The cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 100 units/ml penicillin G, and 100 μg/ml streptomycin at 37°C in a humidified incubator with 5% CO_2.

Methyl Thiazolyl Tetrazolium (MTT) cell viability assay

MGC803, SGC7901 and BGC823 cells were seeded onto 96-well microplate at a density of 2 × 104 cells per well and incubated overnight in 10% FBS medium. The cells were then treated with different concentrations of Z-guggulsterone (0,4,8,12,16,20 and 24 μg/ml) in serum-free conditions. Untreated cells in serum-free medium were used as controls. After incubation for 12, 24 and 48 h at 37°C, the cell proliferation was determined by the MTT assay as described in the previous studies.

Hoechst 33342 staining

MGC803, SGC7901 and BGC823 cells were treated with Z-guggulsterone at the final concentrations of 0,8,12, and 16 μg/ml for 24 h. The cells were then washed twice in PBS followed by fixation with 4% paraformaldehyde for approximately 20 min. After washing three times with PBS, these cells were stained with 10 μg/ml Hoechst 33342 for 10 minutes at room temperature in the dark. Morphological changes in nuclear chromatin were observed under a fluorescence microscope (Eclipse E-800; Nikon, Tokyo, Japan).

Flow cytometry analysis

To further quantitative analysis of apoptosis, the cells were washed with PBS, stained with annexinV-FITC and propidium iodide using the AnnexinV-FITC kit (Dojindo, Japan). The cells were then subjected to flow cytometry according to manufacturer's instructions and the stained cells were analyzed by FACS can flow cytometer.

Western blot analysis

MGC803, SGC7901 and BGC823 cells were grown to subconfluence in 60 mm dishes and thereafter cultured in serum-free medium for 24 h. The cells were then treated with Z-guggulsterone in serum-free conditions for 24 h; untreated cells in serum-free medium were used as controls. In another experiment, the cells were pre-treated with PTEN inhibitor bpv (HOpic, 17.4 ng/ml) or Akt inhibitor MK2206 (38.4 ng/ml) for 30 min and then treated with Z-guggulsterone (16 μg/ml) for 24 h. The extraction of proteins from cells and western blot analyses were performed as described in our previous reports [13,28]. Antibodies used include rabbit anti-Bcl-2, anti-Bax, anti-PTEN, anti-COX-2, and anti-GAPDH (Abcam Inc., Cambridge, MA, USA); anti-phospho-Akt (Ser 473) and anti-Akt (Sigma-Aldrich, St. Louis, MO, USA). Goat anti-rabbit IgG horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from the Abcam Inc. (Cambridge, MA, USA). Bands were quantified using densitometric image analysis software (Quantity One, Bio-Rad, Hercules, CA, USA). The relative expressions of COX-2, PTEN, Bcl-2 and Bax were normalized to that of GAPDH. The phospho-Akt was normalized to the total Akt levels.

Wound-healing assay

Cell migration was assayed using *in vitro* wound healing assay. Gastric cancer cells were plated into the 6-well plates and cultured in incubator until confluent monolayers were formed. The cells were serum starved for 12 h and then a 'wound' (clear space) was made using a sterile pipette tip. At this time point (t=0 h) wound margins were observed using phase contrast microscopy and photographed. Then, serum-free medium containing 0 or 4 μg/ml Z-guggulsterone was added to the plates and the cells were incubated for up to 48 h at 37°C. The same fields of the wound margin were photographed at 24 and 48 h. Pictures were superposed using Photoshop (Adobe) and areas were measured using Scion Image Analysis Software (Scion Corporation, Frederick, MD, USA). The wound healing rate was calculated according to the following formula: (the average area of wound in 0 h-the average area of wound in 24 or 48 h)/the average area of wound in 0 h. Experiments were repeated at least three times.

In vivo antitumor efficacy

Athymic nude mice (4-5 weeks old and weighing 18-20 g, n=30) were purchased from the Department of Laboratory Animal Center of Nanjing Medical University (Nanjing, China). The mice were housed in a specific pathogen-free room with controlled temperature (25 ± 2°C), humidity (60%-80%), and lighting (12 h

light/dark cycle), with autonomous access to water and food. The mice were subcutaneously injected at the left axillary space with 0.1 ml of cell suspension containing 4-6 × 106 MGC803, SGC7901 or BGC823 cells. Seven days after implantation of tumor cells (when the tumor size was approximately 0.1-0.2 cm3), mice were randomly divided into two groups (n=5) to receive different treatments. The experimental mice were administered oral Z-guggulsterone at a dose of 40 mg/kg once a day for 2 weeks. The control mice were administered with an equal volume of normal saline. Tumors were measured with calipers at 2-day intervals and the volumes were calculated using the following formula: (the shortest diameter) 2 × (the longest diameter) × 0.5. The mice were sacrificed at the completion of the experiment and the implanted tumors were removed. All animal experimental procedures were done in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication no. 80-23, revised 1996) and the institutional ethical guidelines for animal experiments.

Statistical analysis

Statistical analyses were performed using the GraphPad Prism 6.0 software (GraphPad Software, Inc., USA). The data were shown as mean ± standard deviation, and were analyzed using two-tailed Student's t-test or one-way analysis of variance (ANOVA) with Dunnett's multiple comparison tests. P-value<0.05 was considered statistically significant.

RESULTS

Effects of Z-guggulsterone on gastric cancer cell proliferation

MTT assay was used to analyze metabolic activity in proliferating cells. As shown in Figure 2, Z-guggulsterone significantly inhibited the cell proliferation of MGC803, SGC7901 and BGC823 in a dose and time-dependent manner. The IC50 of Z-guggulsterone at different time points was shown in Table 1.

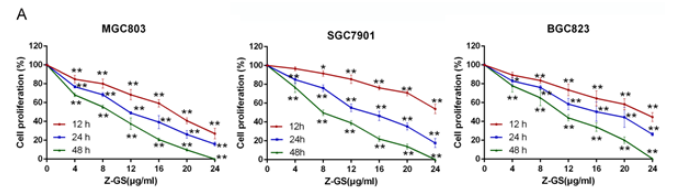

Figure 2: Z-Guggulsterone dose and time-dependently inhibits gastric cancer cell proliferation. The results are expressed as the percentages of the control (0 μg/ml). Data are shown as mean ± SD from three independent experiments. *P<0.05, **P<0.01 vs. control (0 μg/ml).

Table 1: IC50 values of Z-Guggulsterone at 12, 24, and 48 h against MGC803, SGC7901, and BGC823 cells.

	IC50 (μg/ml)		
	12 h	24 h	48 h
MGC803	17.42 ± 1.22	13.09 ± 0.62	9.94 ± 0.13
SGC7901	29.74 ± 2.88	14.79 ± 0.62	10.27 ± 0.38
BGC823	22.87 ± 2.14	16.52 ± 1.61	11.68 ± 0.33
Data are represented as mean ± SD from three independent experiments.			

Effects of Z-guggulsterone on the apoptosis of gastric cancer cells

The morphological changes in apoptotic cells were observed by Hochest 33342 staining. As shown in Figure 3A, the nucleus of untreated control cells were large and round without condensation or fragmentation, whereas the nucleus from the Z-guggulsterone treated cells were condensed and fragmented, emitted bright fluorescence, which were early phenomena of apoptosis. Annexin V-FITC and PI staining were used to evaluate the apoptosis rate, which was reported as the percentage of early apoptotic cells stained with Annexin V (Annexin V+/PI-, Q3) and late apoptotic cells stained with Annexin V and PI (Annexin V+/PI+, Q2). Z-guggulsterone dose-dependently induced apoptosis in MGC803, SGC7901 and BGC823 cells shown in, Figures 3B and 3C. In addition, Z-guggulsterone dose-dependently suppressed the expression of Bcl-2 and increased the expression of Bax in gastric cancer cells shown in Figures 3D and 3E. All these data indicated that Z-guggulsterone induced apoptosis in gastric cancer cell lines.

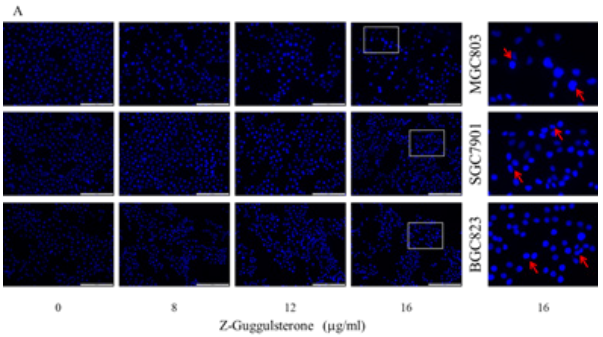

Figure 3A: ZZ-Guggulsterone induces apoptosis in gastric cancer cells. (A) Morphological changes in apoptotic cells were examined by fluorescence microscopy after Hoechst 33342 staining (original magnification, × 100). Morphologic changes were seen only in Z-Guggulsterone treated gastric cancer cells. Magnified images of 16 μg/ml Z-Guggulsterone treated gastric cancer cells clearly showed condensed and fragmented nucleus and emitted bright fluorescence (right panels).

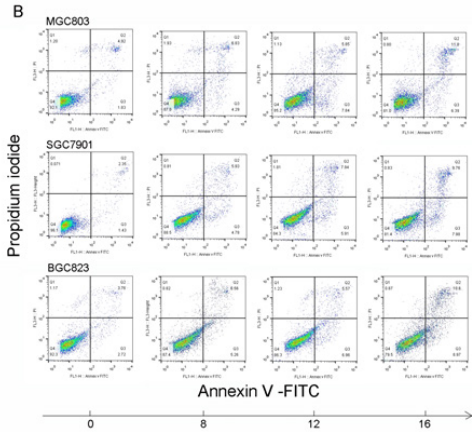

Figure 3B: Flow cytometry-based annexin V-FITC/PI labeling of apoptotic cells.

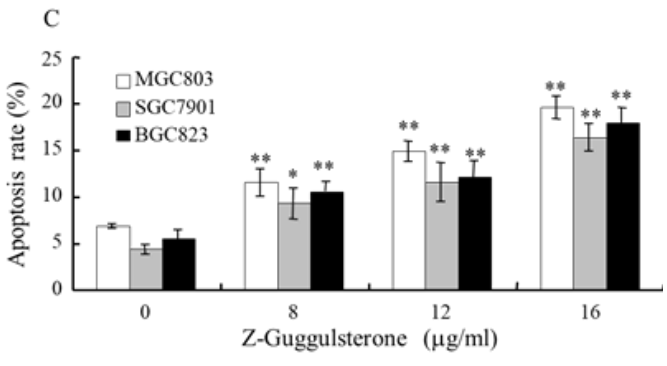

Figure 3C: The histogram represents apoptosis rates.

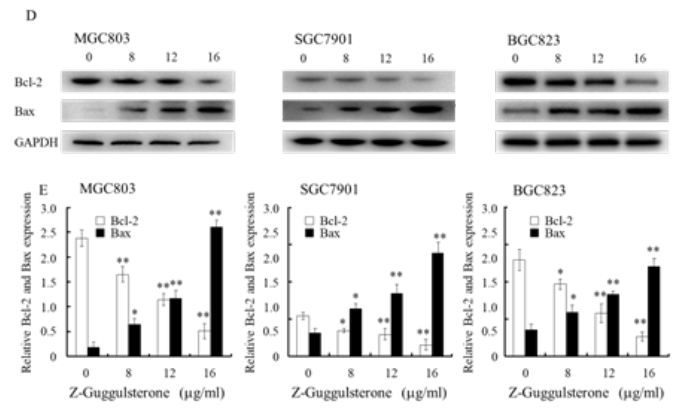

Figure 3 (D and E): The expressions of Bcl-2 and Bax in gastric cancer cells were measured by western blot analysis after Z-Guggulsterone treatment. The histogram represents the relative expressions of Bcl-2 and Bax compared with GAPDH. Each data point represents the mean ± SD from three independent experiments. *P<0.05, **P<0.01 vs. control (0 μg/ml).

Effects of Z-guggulsterone on the expressions of PTEN, COX-2 and p-Akt in gastric cancer cells

FTo examine the effects of Z-guggulsterone on PTEN, COX-2 and p-Akt expression, gastric cancer cell lines were treated with Z-guggulsterone and western blot analysis showed that Z-guggulsterone dose-dependently increased PTEN expression, whereas inhibited COX-2 and p-Akt expressions in MGC803, SGC7901 and BGC823 cells shown in Figure 4.

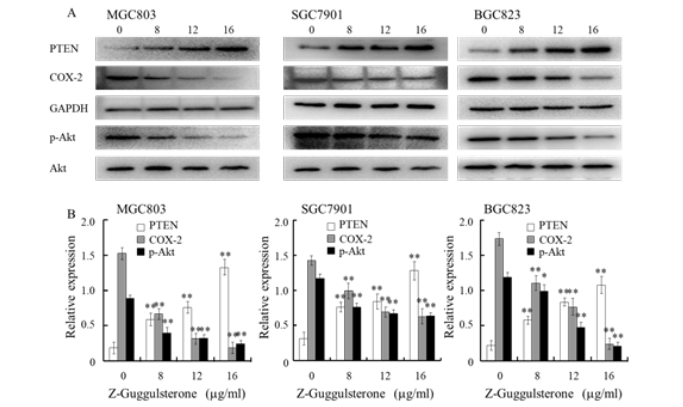

Figure 4: Effects of Z-Guggulsterone on the PTEN and COX-2 expressions as well as Akt phosphorylation in gastric cancer cells. Gastric cancer cells were treated with increasing concentrations of Z-Guggulsterone for 24 h. (A) PTEN, COX-2, total and phosphorylated forms of Akt expressions in gastric cancer cells were detected by western blot analysis. (B) The histogram represents the relative expressions of PTEN and COX-2 compared with GAPDH or phospho-Akt (p-Akt) compared with total Akt, respectively. Each data point represents the mean ± SD from three independent experiments. *P<0.05, **P<0.01 vs. control (0 μg/ml).

Effects of PTEN/Akt inhibitor on Z-guggulsterone -induced downregulation of COX-2 expression in gastric cancer cells

To elucidate the signaling pathway mediating Z-guggulsterone -induced downregulation of COX-2 expression, further studies were designed to determine the effects of PTEN and Akt inhibitor on COX-2 expression in gastric cancer cells. As shown in Figure 5, the PTEN inhibitor, bpv (Hopic), blocked Z-guggulsterone induced PTEN expression, reversed Z-guggulsterone induced downregulation of p-Akt and COX-2 expression. The Akt inhibitor, MK2206, combined with Z-guggulsterone can down-regulate the expression levels of p-Akt and COX-2, and the combined effect was better than that of Z-guggulsterone alone shown in Figure 5C and 5D. These results collectively indicate that Z-guggulsterone inhibits proliferation and induces apoptosis of gastric cancer cells may be mediated by down-regulation of COX-2 expression through PTEN/Akt signaling pathway.

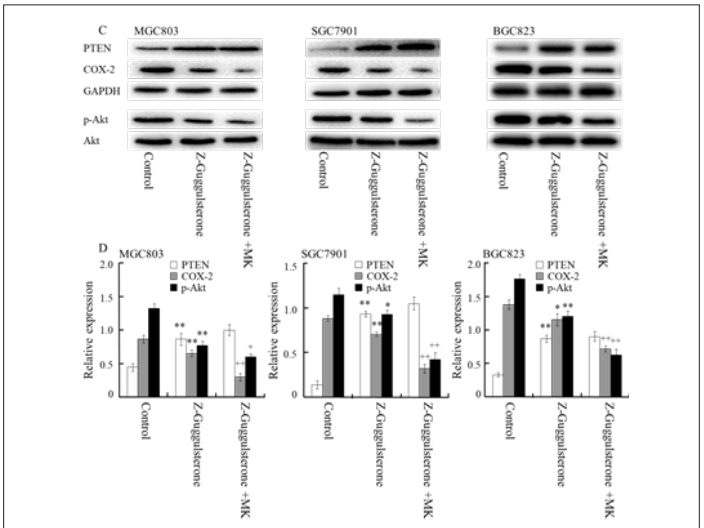

Figure 5: TEffects of PTEN/Akt inhibitor on Z-Guggulsterone-induced downregulation of COX-2 expression in gastric cancer cells. Gastric cells were pre-treated with PTEN inhibitor bpv (HOpic, 17.4 ng/ml, A and B) or Akt inhibitor MK2206 (38.4 ng/ml, C and D) for 30 min and then treated with Z-Guggulsterone (16 μg/ml) for 24 h. (A and C) PTEN, COX-2, total and phosphorylated forms of Akt expressions in gastric cancer cells were detected by western blot analysis. (B and D) The histogram represents the relative expressions of PTEN and COX-2 compared with GAPDH or phospho-Akt (p-Akt) compared with total Akt, respectively. Each data point represents the mean ± SD from three independent experiments. *P<0.05, **P<0.01 vs. Control; +P<0.05, ++P<0.01 vs. ZG (ZG=Z-Guggulsterone).

Z-guggulsterone inhibits the migration of gastric cancer cells

After 24 h and 48 h of Z-guggulsterone treatment, fewer cells migrated to the exposed area of the wound, healing was evidently inhibited. The results indicated that Z-guggulsterone significantly inhibited the migration of gastric cancer cells. As administration time is prolonged, the effect of migration inhibition becomes more evident shown in Figure 6.

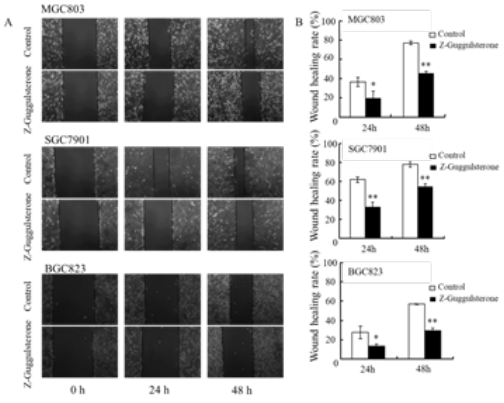

Figure 6: Z-Guggulsterone inhibits monolayer wound healing of gastric cancer cells. (A) Phase micrographs of gastric cancer cells at 24 h and 48 h after monolayer wounding (original magnification, × 100). (B) Quantification of cell migration using the monolayer wound healing assay. Gastric cancer cells were treated without (control) or with 4 μg/ml Z-Guggulsterone. Each data point represents the mean ± SD from three independent experiments. *P<0.05, **P<0.01 vs. control (0 μg/ml).

We selected 4 μg/ml Z-guggulsterone to investigate the effects of Z-guggulsterone on the migration of gastric cancer cells. Z-guggulsterone at this concentration had no obvious influence on cell proliferation according to the data from MTT assay. As shown in Figure 6B, Z-guggulsterone significantly inhibited the migration of MGC803, SGC7901 and BGC823 cells at 24 h and 48 h after treatment.

Z-guggulsterone inhibits gastric xenograft tumor growth *in vivo*

To further explore the effects of Z-guggulsterone on tumor growth, a gastric xenograft tumor model was established. As shown in Figures 7A and 7C, Z-guggulsterone inhibited tumor growth compared to control mice at later time points. There was no significant difference in body weight between experimental and control mice shown in Figure 7B. Furthermore, western blot analysis showed that Z-guggulsterone inhibits COX-2 expression in nude mice xenografts shown in Figures 7D and 7E. These data collectively indicate that Z-guggulsterone can effectively reduce tumor growth by suppressing COX-2 protein expression *in vivo*

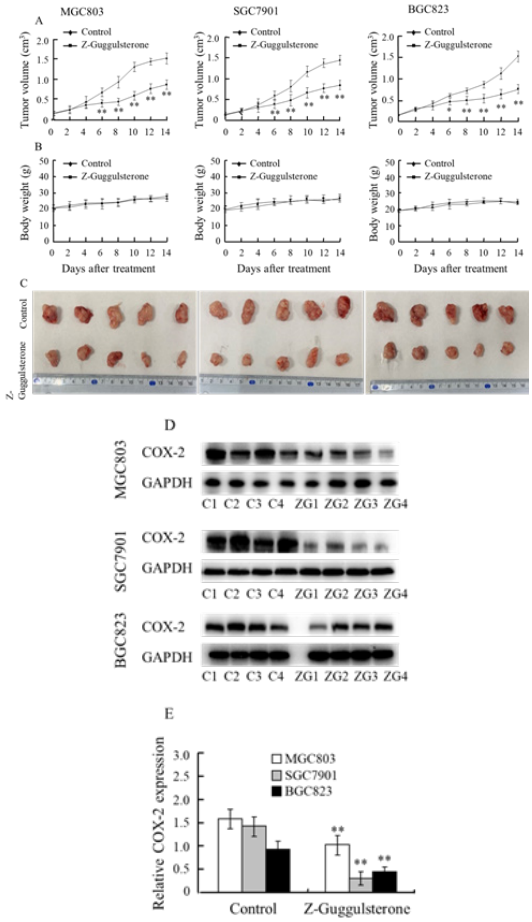

Figure 7: EZ-Guggulsterone inhibits gastric tumor growth *in vivo* . The nude mice were subcutaneously injected with gastric cancer cells. When the tumor size was approximately 0.1-0.2 cm3, mice were randomly divided into two groups (n=5) to receive different treatments. The experimental mice were administered oral Z-Guggulsterone at a dose of 40 mg/kg once a day for 2 weeks. The control mice were administered with an equal volume of normal saline. (A) Tumor volume was measured and the tumor growth curves were drafted. (B) Body weight change of mice treated with saline or Z-Guggulsterone. (C) The mice were sacrificed at the completion of the experiment and each tumor lump was removed from the body. (D) Western blot analysis of COX-2 expression in the xenografts obtained from four randomly selected mice. (C=control; ZG=Z-Guggulsterone). (E) The histogram represents the relative expression of COX-2 proteins compared with GAPDH. Data were shown as mean ± SD from five (A and B) or four mice (E). *P<0.05, **P<0.01 vs. control (saline).

DISCUSSION

Gastric cancer remains as the primary cause of cancer-related deaths worldwide, despite the fact that the incidence has gradually declined in developed countries [3]. Over-expression of COX-2 plays an important role in the carcinogenesis, development, and progression of gastric cancer [8,10-12,30]. Specific COX-2 inhibitors can effectively inhibit the proliferation and induce the apoptosis of gastric cancer cells [13,31]. In this study, we demonstrated for the first time that Z-guggulsterone can down-regulate the expression of COX-2 via the PTEN/Akt signaling in gastric cancer cells, and consequently induces apoptosis and inhibits the proliferation of gastric cancer cells.

Z-guggulsterone is a resinous substance obtained from Commiphora trees, which is believed to act as an antitumor agent [22,32,33]. Previous studies have confirmed that guggulsterone can reduce COX-2 expression level in head and neck cancer cells [34] and digestive tract tumor cells [35,36]; prevented cytokines-induced COX-2 expression and protects pancreatic beta cells from cytokine toxicity [37]. The results of these studies are consistent with our findings that Z-guggulsterone can significantly inhibit COX-2 expression in gastric cancer cells. However, the molecular mechanism by which Z-guggulsterone down-regulates the expression of COX-2 in gastric cancer cells remains unknown.

It is well known that tumorigenesis is due to an imbalance between cell proliferation and apoptosis, which is maintained by different signal transduction pathways. In the current study, we have observed that Z-guggulsterone significantly inhibited cellular proliferation and migration, induced apoptosis in vitro, as well as inhibited tumor growth *in vivo* . In addition, Z-guggulsterone significantly inhibited the expression of Bcl-2, p-Akt and COX-2 as well as increased Bax and PTEN expression in gastric cancer cells.

The PTEN inhibitor, bpv (Hopic), blocked Z-guggulsterone -induced PTEN expression, reversed Z-guggulsterone -induced downregulation of p-Akt and COX-2 expression. The Akt inhibitor, MK2206, combined with Z-guggulsterone can down-regulate the expression levels of p-Akt and COX-2, and the combined effect was better than that of Z-guggulsterone alone. These results collectively indicate that Z-guggulsterone inhibits proliferation and induces apoptosis of gastric cancer cells may be mediated by down-regulation of COX-2 expression through PTEN/Akt signaling pathway. Antitumor effect of a selective COX-2 inhibitor may be attributed to angiogenesis inhibition through modulating the PTEN/PI3K/Akt/HIF-1 pathway in murine hepatocarcinoma model [38].

A large number of studies have confirmed that the apoptosis of cancer cells can be induced by the PI3K/Akt pathway [39-42]. The antitumor effect of the traditional Chinese medicine ginsenoside is related to its inhibition of the PI3K/Akt signaling pathway and COX-2 expression [43]. It has been previously shown that PTEN/Akt pathway may reduce tumor growth and metastasis in breast cancer [44,45]. Cadmium selectively induces COX-2 expression through PTEN-mediated PI3K/Akt activation In RAW 264.7 cells, a mouse leukaemic monocyte cell line [46]. PTEN is known to negatively regulate PI3K signaling pathway due to its lipid phosphatase activity, thereby inhibiting the activation of downstream components such as AKT [47-49]. Previous studies have shown that PTEN-mediated tumor inhibition is related to the suppression of PI3K/AKT pathway, which is abnormally activated in human cancer [16,50,51] (Figure 8).

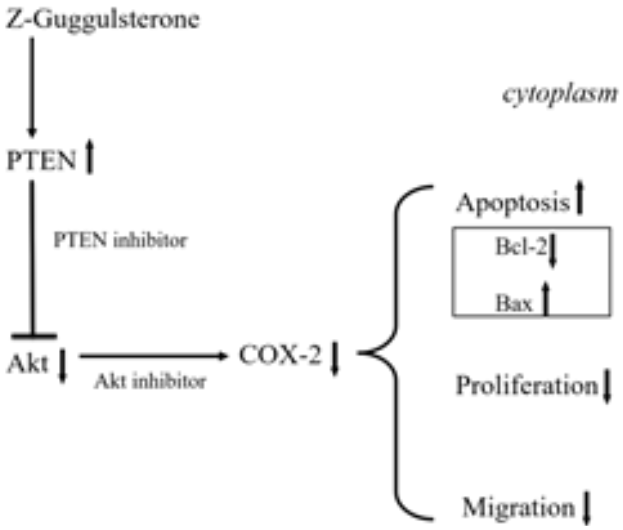

Figure 8: A hypothetical model illustrating that Z-Guggulsterone inhibits the proliferation and migration and induces apoptosis through the PTEN/Akt/COX-2 signaling pathway in gastric cancer cells.

CONCLUSION

In conclusion, we have demonstrated that Z-guggulsterone significantly inhibited cellular proliferation, migration and induced apoptosis in vitro, as well as inhibited tumor growth in *vivo* . Z-guggulsterone increased Bax and PTEN expression as well as inhibited the expression of Bcl-2, p-Akt and COX-2 in gastric cancer cells. In addition, PTEN inhibitor significantly reversed Z-guggulsterone -induced down regulation of p-Akt and COX-2 expression. These results suggest that Z-guggulsterone inhibits proliferation and induces apoptosis of gastric cancer cells may be mediated by down-regulation of COX-2 expression through PTEN/Akt signaling pathway.

AUTHOR'S CONTRIBUTION

M.S. and G.L. performed the *in vitro* and *in vivo* experiments. J.W. and J.H. assisted with the cell based assays. P. H. and S.W. performed the data analysis. Y.L. helped perform the analysis with constructive discussions. W.S. and X.H. supervised the overall experimental design, provided the idea, and prepared the manuscript.

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DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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