Involvement of Bcl-2, Src, and ERα in gossypol-mediated growth inhibition and apoptosis in human uterine leiomyoma and myometrial cells

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Aim: To investigate the effect of gossypol on the growth of cultured human uterine leiomyoma and myometrial cells, the level of Bcl-2 and the activity of Src and estrogen receptor (ERα).

Methods: Human uterine leiomyoma and adjacent normal myometrial cells were cultured in vitro. Both cell types were treated with a graded concentration of gossypol. Cell viability was assayed using CCK-8. Morphological change was observed with optical and electronic microscopy. Apoptosis was evaluated using TUNEL assay. Levels of Bcl-2, ERα and Src were analyzed using Western blotting.

Results: Gossypol significantly inhibited growth and promoted apoptosis in cultured human uterine leiomyoma cells with the IC50 value and its corresponding 95% confidence intervals (CI) of 6.5 (4.0–10.5), 9.0 (4.9–16.5), and 7.5 (4.0–14.1) μmol/L at 20, 40, and 60 h, respectively. Gossypol exerted inhibitory effects on the myometrial cells with the IC50 value and its 95% CI of 49.1 (28.3–85.0), 14.5 (7.7–27.4), and 2.6 (1.2–5.6) μmol/L at 20, 40, and 60 h, respectively. Compared with control, gossypol 0.1-3.0 μmol/L markedly decreased the protein expression of Bcl-2 (P<0.05) in both leiomyoma and myometrial cells in a concentration-dependent manner, and significantly suppressed the level of phospho-Tyr416Src (P<0.05) in both cell types at 3.0 μmol/L without obvious alteration of c-Src and phospho-Tyr527Src levels (P>0.05). In addition, gossypol markedly reduced both the expression of ERα (P<0.05) at the low concentration of 0.1 μmol/L in the myometrial cells and the level of phospho-ser167ERα (P<0.05) at the high concentration of 3.0 μmol/L in the leiomyoma cells.

Conclusion: Gossypol inhibits proliferation and induces apoptosis in human uterine leiomyoma and myometrial cells. It is likely that the mechanisms of action involve reducing the protein level of Bcl-2 and the activity of Src and ERα.

Keywords: gossypol; uterine leiomyoma; myometrium; apoptosis; Bcl-2; c-Src; estrogen receptor α

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Introduction

Uterine leiomyoma (fibroid or myoma) is a common benign tumor in premenopausal women. It occurs in more than 70% of American women of reproductive age[1], and there is also a high occurrence in China. It is generally accepted that increased serum levels of estradiol (E2) and progesterone (P) and high expression of estrogen receptors (ERs) and progesterone receptors (PRs) act as predominant promoters in the development of the leiomyoma. A recent study shows that cytokines and growth factors are over-expressed in uterine leiomyoma compared with normal myometrium[2]. These findings suggest that there are intricate pathways involved in the formation of the leiomyoma.

It is likely that an anti-apoptotic mechanism is involved in the development of uterine leiomyoma. Bcl-2 protein was reported to be over-expressed in leiomyomas when compared with homologous myometrium. Expression of Bcl-2 protein showed a strong correlation to ERs in leiomyoma[3, 4]. Estrogen inhibits apoptosis in several cell types by inducing Bcl-2 expression[5, 6], and an anti-estrogen reagent induced apoptosis by down-regulating the expression of Bcl-2 protein[7].

Moreover, c-Src, a member of the Src family of non-receptor protein tyrosine kinases, may also play a role in the formation of uterine leiomyoma. Src family kinases are involved in a number of signal transduction pathways[8]. Activation of Src family kinases protects cells from apoptosis[9] whereas...
inhibition of Src tyrosine kinase reduces mitosis and induces apoptosis. Recent evidence indicates that there is an important feed-forward signaling loop involving E2, ERα, and Src[10]. Classical sex steroid receptors (ERs, PRs) can activate Src, and Src can drive expression of certain ERα target genes. Overexpression and aberrant activation of c-Src have been found in a variety of epithelial and non-epithelial cancers[11, 12]. We demonstrated that c-Src was also over-expressed in a guinea pig model of uterine leiomyoma induced by estradiol[13]. This evidence suggests that Src may be involved in the development of leiomyoma.

Gossypol, a product of the cotton plant, was initially investigated as an oral contraceptive for males[14, 15]. However, the anti-fertility research was abandoned because of unacceptably high rates of permanent infertility. There are, however, many studies on gossypol are still in progress. Gossypol has demonstrated potent anti-proliferative properties in vitro and in vivo[16–20], and it is considered a promising anti-cancer candidate. In Chinese suburban hospitals, there has been interest in applying gossypol in the treatment of gynecological disease, such as endometriosis and uterine leiomyomas, because it is cheap and easily available. A preliminary clinical study showed that the volume of uterine leiomyoma was significantly reduced after patients were administered gossypol (10–20 mg/d) for 3 months[21, 22]. Unfortunately, there is a lack of sufficient experimental data to support this, and it is important to confirm gossypol’s effectiveness in suppressing the growth of uterine leiomyoma before it is widely used. Therefore, in this study, we investigated the effect of gossypol on the growth of human uterine leiomyoma in vitro and the levels of Bcl-2, estrogen receptor alpha (ERα), Src and its active form to explore the possible mechanism of action. In addition, we also investigated the effect of gossypol on the adjacent normal myometrial cells.

Materials and methods
Chemicals and reagents
Gossypol, C_{30}H_{30}O_{8} (molecular weight 518.55, purity>98%) was a kindly gift of Prof ZHANG (Dongwu University, Jiangsu Province, China). The structure of gossypol is shown in Figure 1. PP2 (4-amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazolo-3,4-d] pyrimidine) was purchased from Calbiochem (EMD Biosciences, Inc, Merck KGaA, Darmstadt, Germany). Dextrancoated-charcoal treated fetal calf serum (D-FCS) was obtained from Biological Industries Ltd (Kibbutz Beit Haemek, Israel).

![Figure 1. Structure of gossypol.](image)

DMEM-F12 (1:1) and HEPES were purchased from Gibco, Invitrogen Co (Carlsbad, CA, USA). Dulbecco’s modified Eagle’s medium (DMEM): Nutrient Mixture F-12 (DMEM/ F-12) Media and Hank’s Balanced Salt Solution (HBSS), DMSO and type II collagenase were obtained from Sigma Chemical Co (St Louis, MO, USA). CCK-8 kit was obtained from Dojindo Molecular Technologies (Kumamoto, Japan). FragEL DNA fragmentation detection kit was obtained from Calbiochem (EMD Biosciences, Inc, Merck KGaA, Darmstadt, Germany). Mouse anti-ERα monoclonal antibody (65 kDa), c-Src rabbit polyclonal antibody (60 kDa), anti-phospho-Tyr416Src mouse monoclonal antibody (60 kDa), anti-phospho-Tyr527Src mouse monoclonal antibody (60 kDa) and Bcl-2 rabbit polyclonal antibody (26 kDa) were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-phospho-ERα (Ser167) antibody (66 kDa) was obtained from Upstate Cell Signaling Solution (Lake Placid, NY, USA). The enhanced chemiluminescence (ECL) detection kit and bicinchoninic acid (BCA) kit were purchased from Pierce (Rockford, IL, USA). Dharacon siRNA transfection reagents, siCONTROL Non-Targeting siRNA Pools and siGENOME ON-Targeting SMART pool were purchased from Thermo Fisher Scientific Inc (MA, USA).

Tissue collection
The leiomyoma and adjacent normal myometrial tissue samples were obtained from premenopausal women (38–45 years old) with regular menstrual cycles who were undergoing hysterectomies. None of the patients had received any hormonal therapy before surgery. Patients underwent surgery in 2008–2009 at Shanghai Gynecological and Obstetrics Hospital. Informed consent for the use of the tissue was obtained from each patient before surgery. The study was approved by the Ethical Committee for Clinical Research of the Hospital and Shanghai Institute of Planned Parenthood Research. The histological diagnosis of each uterine specimen was examined.

Samples were collected from the scheduled surgeries at the patient’s convenience during the proliferative phase of the menstrual cycle.

Cell culture and treatment
The culture of leiomyoma and adjacent normal myometrial cells was performed as follows: each leiomyoma and myometrial tissue sample was rinsed in Ca^{2+}- and Mg^{2+}-free HBSS, cut into small pieces, and then digested in pre-warmed HBSS containing 2.0% type II collagenase (w/v) and 25 mmol/L of HEPES at 37 °C for 4–5 h. The cells were collected by centrifugation at 460×g for 5 min and washed several times with HBSS. The isolated leiomyoma cells and myometrial cells were then transferred to a 75-cm² flask at a density of 1×10⁶ cells/mL and cultured in phenol-red free DMEM-F12 (1:1) with 10% (v/v) D-FCS, 25 mmol/L HEPES (pH 7.4), 2 mmol/L glutamine, 1 mmol/mL sodium pyruvate, 10 mmol/mL non-essential amino acids and 1 mmol/mL insulin-transferrin-selenium-G supplement. The cells were cultured in a humidified atmosphere of 5% CO₂/95% air. The culture medium was replaced every 3–4 d until the cells reached confluence (10–12 d).
d). The cultured cells were then digested with 0.25% trypsin and subcultured under the same conditions as the primary cultures. The primary and subcultured smooth muscle cells were confirmed by immunohistochemistry staining of the muscle-specific protein desmin and α-smooth muscle actin.

At approximately 70% confluence, the myometrial and leiomyoma cell cultures were treated with gossypol or DMSO alone as a control. Gossypol was dissolved in DMSO and diluted to a desired concentration. The final concentration of DMSO in culture media was 0.5% (v/v).

Cell viability assay
The viabilities of cultured leiomyoma and myometrial cells were determined by WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] assay using cell-counting kit-8 (CCK-8). The cells were cultured in 96-well plates and treated with DMSO or graded concentrations of gossypol (0.01, 0.05, 0.1, 0.5, 1.0, 5.0, 10, or 50 μmol/L) for 20, 40, and 60 h. Ten μL CCK-8 was added to each well, and cells were incubated at 37 °C for 2 h. Using a spectrophotometric plate reader (Bio-Tek ELX-800, USA), the absorbance (OD) at 450 nm was read to determine cell viability in each well. Results are presented as the percentage of cell growth inhibition rate (%) and the half maximal inhibitory concentration (IC50). Control cells were not treated with gossypol, and the cell growth inhibition rate (%) of cells treated with gossypol was calculated by the following equation: [(1-OD of gossypol-treated wells/OD of control wells)×100%]. The IC50 was calculated from the dose response using the Bliss method. The experiments were performed in three different samples.

Transmission electron microscopy
Morphological changes in the leiomyoma and myometrial cells were observed by electron microscopy. Briefly, the cells treated with DMSO or graded concentrations (0.1, 1.0, and 3.0 μmol/L) of gossypol were collected and then stained by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) using the TdT-FragEL DNA fragmentation detection kit. Briefly, the adherent cells were cultured and fixed in a 96-well Nunc plate. Then, we performed the experiment according to the manufacturer’s instructions. The cells were stained with DAB, and the numbers of stained positive cells were observed using a light microscope. A negative control was generated by substituting dH2O for the TdT in the reaction mixture during the labeling step. The present values were an average ratio of the number of positive cells against total cells in three different samples.

Western blinding
Western blotting was used to evaluate the levels of Bcl-2, ERα and Src and its activity. Cells treated with DMSO or graded concentrations (0.1, 1.0, and 3.0 μmol/L) of gossypol were collected. Western blotting was performed according to routine procedure. The protein concentration was determined using the bicinchoninic acid (BCA) method according to the manufacturer’s instructions. Fifty-μg samples were loaded onto 10% SDS-PAGE (30% acrylamide:bisacrylamide 29:1) gels for electrophoresis. The primary antibodies for Bcl-2, ERα, Src, phosphorylated-167ER, phosphorylated-416Src, and phosphorylated-527Src were diluted at 1:3000. The primary β-actin antibody was diluted at 1:10000. The relative levels of protein were semiquantitatively determined with Image-Pro Plus® Version 5.1.0 software (Media Cybernetics Inc). The relative levels of protein were obtained by taking the ratio of the band intensity of the target protein against that of β-actin. Statistical analysis was performed on the average protein level of three samples.

Src siRNA transfection
Specific siRNA oligonucleotides against Src and control scrambled siRNA oligonucleotides were transfected into the leiomyoma and myometrial cells cultured in media with 5% FBS using Dharmacon siRNA transfection reagents for 4 h. The cells were transferred to media containing D-FCS for 36 h and then collected for Western blot analysis to determine the levels of ERα and Src.

Statistical analysis
The data are presented as mean±SD. The statistical significance of the inhibition rate at various times was determined by repeated measurement by ANOVA with SPSS 11.5 software (version 11.5 for Windows; SPSS, Chicago, Illinois, USA), and then a paired-sample t test was used between 2 groups. Other statistical significance among 3 or more groups was determined by one-way AVONA. P<0.05 was considered statistically significant.

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ber of viable cultured leiomyoma and myometrial cells were determined by CCK-8 assay and then presented as the percentage of viable cells inhibition rate and IC$_{50}$. In the myometrial cells, the IC$_{50}$ of gossypol and its corresponding 95% confidence intervals was $6.5$ (4.9–10.5), $9.0$ (5.0–16.5), and $7.5$ (4.0–14.1) μmol/L. There was no pronounced difference in the inhibition rates of viable cells observed among 20, 40, and 60 h ($P$$>$0.05). In the leiomyoma cells, the IC$_{50}$ of gossypol and its corresponding 95% confidence intervals was $49.1$ (28.3–85.0), $14.5$ (7.7–27.4), and $2.6$ (1.2–5.6) μmol/L at 20, 40, and 60 h, respectively. There was a significant difference between the inhibition rates of viable cells detected between 20 and 60 h ($P$$<$0.05, Figure 2).

**Effects of gossypol on the morphological change of cultured uterine leiomyoma and myometrial cells**

The morphological changes of both cell types were observed via optical and electronic microscope. Under the optical microscope, both cell types treated with DMSO appeared robust, with an elongated shape and abundant cytoplasm. The leiomyoma cells were morphologically similar to the myometrial cells, but they lined up more densely and exhibited a whirlpool or fan-shape. In the presence of gossypol, both cell types appeared thin, and the cytoplasm atrophic and intercellular connection dwindled (Figure 3).

Under the electron microscope, a series of distinct morphological changes in both cell types was observed after treatment with a graded concentration of gossypol. Both cell types showed extensive cell death with features of apoptosis. The cells maintained membrane integrity but showed signs of cell shrinkage, nuclear deformation, increased cytoplasmic vacuolar degeneration, slight aggregation of chromatin, and damage of the cytoplasmic organelles (e.g., mitochondrial swelling, the disappearance of mitochondrial cristae and enlargement of the endoplasmic reticulum) (Figure 4).

**Gossypol-induced apoptosis in the cultured uterine leiomyoma and myometrial cells**

Apoptosis was assessed by TUNEL. Non-apoptotic cells were predominantly rounded and appeared counterstained, and...
apoptotic cells appeared dark brown with a variety of distinctive morphological changes, including pyknosis, dark brown encircling, and dense-stained oval, crescent or ring-shaped nuclei or granules. In the presence of gossypol, there were more dark brown-stained cells observed in both leiomyoma and myometrial cells compared with the control cells. Compared with the control, there was a significant increase in the ratio of apoptotic cells after treatment with 3.0 μmol/L gossypol in both cell types (P<0.05, Figure 5).

Effects of gossypol on the expression of Bcl-2 in uterine leiomyoma and myometrial cells

We further investigated the effect of gossypol on Bcl-2 expression to analyze the mechanism of apoptosis induction.
results indicated a small increase in the expression of Bcl-2/β-actin in the leiomyoma in comparison to the myometrial cells, but no obvious difference was detected between the two types of cells \((P>0.05)\). Gossypol inhibited the protein level of Bcl-2/β-actin in both cells in a concentration-dependent manner. Compared with the control, there was a pronounced decline in the expression of Bcl-2/β-actin in both cell types after treatment with 1.0 or 3.0 μmol/L gossypol \((P<0.05, \text{Figure } 6)\).

**Effects of gossypol on the level of ERα and phospho-ser167ERα in uterine leiomyoma and myometrial cells**

The effect of gossypol on the levels of ERα and phospho-ser167-ERα were analyzed by Western blotting. The results showed a small increase in the expression of ERα/β-actin in the leiomyoma cells in comparison with the myometrial cells, but no pronounced difference was detected between the two cell types \((P>0.05)\). Gossypol inhibited the expression of ERα/β-actin in both leiomyoma and myometrial cells at 0.1 μmol/L, and there was a significant difference between the control and myometrial cells \((P<0.05, \text{Figure } 7)\).

One active form of ERα is phosphorylated at serine-167. The results indicated a small increase in the level of phospho-ser167ERα/β-actin in the leiomyoma cells when compared with the myometrial cells, but no pronounced difference was

![Figure 5. Effects of gossypol on apoptosis of the cultured uterine leiomyoma and myometrial cells. Both cells were cultured in the presence of DMSO or gossypol for 40 h. The apoptosis were detected using TUNEL method. Apoptotic cells with markedly circled, oval, crescent or ring-shape dense-stained nucleus or granule were easily distinguishable from non-apoptotic cells. (A) Myometrial cells treated with DMSO alone (×200). (B) Myometrial cells treated with gossypol 0.1 μmol/L (×200). (C) Myometrial cells treated with gossypol 1.0 μmol/L (×200). (D) Myometrial cells treated with gossypol 3.0 μmol/L (×200). (E) Leiomyoma cells treated with DMSO alone (×200). (F) Leiomyoma cells treated with gossypol 0.1 μmol/L (×200). (G) Leiomyoma cells treated with gossypol 1.0 μmol/L (×200). (H) Leiomyoma cells treated with gossypol 3.0 μmol/L (×200). Data are presented as means±SD of three independent samples. \(bP<0.05\) vs control cells; \(eP<0.05\) vs gossypol 0.1 μmol/L (G-0.1) treated cells; \(hP<0.05\) vs gossypol 3.0 μmol/L (G-3.0) treated cells.](image)

![Figure 6. Effects of gossypol on the protein expression of Bcl-2 in the cultured leiomyoma and myometrial cells, as assessed by Western blotting. Both cells were cultured in DMSO (as control) or graded concentrations of gossypol (at 0.1, 1.0, and 3.0 μmol/L) for 40 h. Data was presented as means±SD of three independent samples. \(bP<0.05\) vs control cells; \(eP<0.05\) vs gossypol 0.1 μmol/L (G-0.1) treated cells; \(hP<0.05\) vs gossypol 3.0 μmol/L (G-3.0) treated cells.](image)
detected between the two cell types \((P>0.05)\). In the presence of gossypol, there was a concentration-dependent decline in the level of phospho-ser167ERα/β-actin in both cell types, and a pronounced difference was detected in the leiomyoma cells compared with the control cells \((P<0.05)\) at a concentration of 3.0 μmol/L (Figure 7).

**Effects of gossypol on the levels of c-Src, phospho-Tyr416Src, and phospho-Tyr527Src in leiomyoma and myometrial cells**

The levels of Src, phospho-Tyr416Src and phospho-Tyr527Src in the leiomyoma and myometrial cells were analyzed by Western blotting. The results indicated a minor increase in the level of c-Src/β-actin, phospho-Tyr416Src, and phospho-Tyr527Src in the leiomyoma cells in comparison with the myometrial cells, but no significant difference was detected between the two cell types \((P>0.05)\). Compared with the control, there was no significant change detected in the levels of c-Src/β-actin and phospho-Tyr527Src in both cell types after treatment with 0.1–3.0 μmol/L gossypol \((P>0.05)\). However, there was a concentration-dependent decline in the expression of phospho-Tyr416Src in both cell types, and there was a pronounced decrease observed in both cell types at 3.0 μmol/L compared with control cells \((P<0.05, \text{Figure 8})\).

**The effect of Src inhibition on the growth of leiomyoma and myometrial cells**

To determine whether Src kinase indeed plays a role in the growth of uterine leiomyoma and myometrial cells, we examined the effects of PP2, a specific Src inhibitor on both cell types. PP2 (0.66 and 6.6 μmol/L) inhibited the growth of leiomyoma and myometrial cells and decreased the levels of c-Src/β-actin and phospho-Tyr527Src in both cell types in a concentration-dependent manner. There was a significant difference in the levels of c-Src/β-actin and phospho-Tyr416Src in the control in comparison with the PP2 treated cells \((P<0.05)\). In addition, PP2 slightly increased the expression of ERα/β-actin and phospho-ser167ERα/β-actin in myometrial cells \((P>0.05)\) compared with control (Figures 2, 7, and 8).

We further examined the specific role of Src in the leiomyoma and myometrial cells with Src siRNA. The growth of the leiomyoma and myometrial cells was impaired after treatment with Src siRNA (12.5, 25, and 50 nmol/L). In comparison with the control siRNA, reduced expression of Src and increased expression of ERα were observed in both Src siRNA-transfected cell types (Figure 9).
Discussion

In the present study we demonstrated that gossypol inhibits growth and induces apoptosis in human uterine leiomyoma cells in vitro in a concentration- and time-dependent manner. The results support the therapeutic efficacy of gossypol in the treatment of uterine leiomyoma. Gossypol has inhibitory effects on myometrial cells as well. The mechanism of action involves the suppression of Bcl-2 expression and decreasing Src activity at a higher concentration. In addition, gossypol significantly reduces both the expression of ERα at a low concentration in the myometrial cells and the activity of ERα at a high concentration in the leiomyoma cells.

Most commonly, a leiomyoma is a partial lesion of the myometrium. Considering that gossypol may be administered to those who suffer from small leiomyomas and want to become pregnant, it is necessary to investigate the effects of gossypol on both leiomyoma and the adjacent normal myometrium. By comparing the IC50 values and the percentage of inhibition rate, we found that gossypol exerts different inhibitory effects on the leiomyoma and myometrial cells. In the myometrial cells, the inhibition rates at various times from 20 to 60 h were not noticeably different. However, gossypol caused a markedly stronger inhibition of the growth of leiomyoma cells at 60 h than at 20 and 40 h. At 20 h, a higher concentration of gossypol was required to inhibit the growth of leiomyoma cells than was required to inhibit the growth of myometrial cells. The results suggest that there was less response to gossypol in the leiomyoma cells than that in the myometrial cells before 40 h. It may imply that, clinically, more time is required for gossypol to effectively reduce the volume of the leiomyoma. In addition, the results suggest that gossypol suppresses not only the proliferation of leiomyoma cells but also the growth of adjacent myometrial cells, both of which might help to reduce the formation of leiomyoma. On the other hand, the findings also suggested that gossypol would lead to an adverse effect on the growth of normal myometrium.

Figure 8. Effects of gossypol on the levels of c-Src, phospho-Tyr416 Src, and phospho-Tyr527 Src in cultured leiomyoma and myometrial cells, as assessed via Western blotting. Both cells were cultured in DMSO (as control) or graded concentrations of gossypol (0.1, 1.0, and 3.0 μmol/L) for 40 h. Data were presented as means±SD of three independent samples. *P<0.05 vs control cells; †P<0.05 vs gossypol 0.1 μmol/L (G-0.1) treated cells; ‡P<0.05 vs gossypol 1.0 μmol/L (G-1.0) treated cells; §P<0.05 vs gossypol 3.0 μmol/L (G-3.0) treated cells; ¶P<0.05 vs PP2 0.66 μmol/L (PP2-0.66) treated cells; ‖P<0.05 vs PP2 6.6 μmol/L (PP2-6.6) treated cells.
With electron microscopy and the TUNEL assay, we confirmed that gossypol induces apoptosis in a concentration-dependent manner in both cell types. The apoptotic characteristics induced by gossypol were similar to those previously reported in spermatocytes and cancer cells\[23, 24\]. To explore the mechanism of apoptosis induction by gossypol, we investigated the expression of Bcl-2 in both cell types. Bcl-2 has already been recognized as an inhibitor of the apoptotic process. Related to sex steroid hormones, Bcl-2 protein expression in leiomyoma cells has been extensively reported\[25\]. In this study, we noticed that there was no significant difference in Bcl-2 expression in the leiomyoma and myometrial cells, and this is supported by data previously reported by Wu et al\[3\].

Gossypol induces apoptosis associated with decreased expression of Bcl-2. In fact, gossypol has also been identified as a potent Bcl-2/Bcl-X\textsubscript{L} small molecule inhibitor in other tumors and spermatic cells\[24, 26\]. Our findings are similar to those in malignant tumor cells.

Recent evidence shows there is a correlation between the activity of members of the Bcl-2 family and c-Src. c-Src tyrosine kinase plays an important role in the regulation of the cell cycle and cell proliferation\[8\]. Src acts not only as a potent mitogenic signaling element but also as an antiapoptotic signaling protein\[27, 28\]. Activation of Src family kinases plays a significant role in enhanced Bcl-2 expression\[29\], and the inhibition of Src reduces the expression of Bcl-2 and Bcl-X\textsubscript{L} and induces apoptosis\[30–32\]. Since gossypol induces apoptosis as an inhibitor of Bcl-2, we investigated the impact of gossypol on the expression of Src. In a preliminary immunohistochemical experiment, we found that c-Src was expressed in the cultured human leiomyoma cells (data not shown). Here, we demonstrated that gossypol has no obvious impact on the expression of c-Src in both cell types. We speculate that apoptosis induced by gossypol is not directly correlated with the protein expression of Src.

To verify that the inhibition of Src kinase indeed plays a role in the gossypol-mediated cell death, we also examined the effects of Src siRNA and PP2, a specific Src inhibitor, on both cell types. In another study, PP2 was shown to induce apoptosis in both cell types (data not shown). Here, we demonstrated that the growth of both cell types was suppressed by either PP2 or Src siRNA in a concentration-dependent manner.

With electron microscopy and the TUNEL assay, we confirmed that gossypol induces apoptosis in a concentration-dependent manner in both cell types. The apoptotic characteristics induced by gossypol were similar to those previously reported in spermatocytes and cancer cells\[23, 24\]. To explore the mechanism of apoptosis induction by gossypol, we investigated the expression of Bcl-2 in both cell types. Bcl-2 has already been recognized as an inhibitor of the apoptotic process. Related to sex steroid hormones, Bcl-2 protein expression in leiomyoma cells has been extensively reported\[25\]. In this study, we noticed that there was no significant difference in Bcl-2 expression in the leiomyoma and myometrial cells, and this is supported by data previously reported by Wu et al\[3\]. Gossypol induces apoptosis associated with decreased expression of Bcl-2. In fact, gossypol has also been identified as a potent Bcl-2/Bcl-X\textsubscript{L} small molecule inhibitor in other tumors and spermatic cells\[24, 26\]. Our findings are similar to those in malignant tumor cells.

Recent evidence shows there is a correlation between the activity of members of the Bcl-2 family and c-Src. c-Src tyrosine kinase plays an important role in the regulation of the cell cycle and cell proliferation\[8\]. Src acts not only as a potent mitogenic signaling element but also as an antiapoptotic signaling protein\[27, 28\]. Activation of Src family kinases plays a significant role in enhanced Bcl-2 expression\[29\], and the inhibition of Src reduces the expression of Bcl-2 and Bcl-X\textsubscript{L} and induces apoptosis\[30–32\]. Since gossypol induces apoptosis as an inhibitor of Bcl-2, we investigated the impact of gossypol on the expression of Src. In a preliminary immunohistochemical experiment, we found that c-Src was expressed in the cultured human leiomyoma cells (data not shown). Here, we demonstrated that gossypol has no obvious impact on the expression of c-Src in both cell types. We speculate that apoptosis induced by gossypol is not directly correlated with the protein expression of Src.

To verify that the inhibition of Src kinase indeed plays a role in the gossypol-mediated cell death, we also examined the effects of Src siRNA and PP2, a specific Src inhibitor, on both cell types. In another study, PP2 was shown to induce apoptosis in both cell types (data not shown). Here, we demonstrated that the growth of both cell types was suppressed by either PP2 or Src siRNA in a concentration-dependent manner. This indicates that c-Src plays a vital role in the growth of leiomyoma cells, and the inhibition of c-Src may be effective in uterine leiomyoma therapy.

Because c-Src is a tyrosine kinase, the regulation of its activity is very important. Tyrosine phosphorylation regulates activity or inactivity at different sites. Phosphorylation of a conserved tyrosine in the activation loop (Tyr-416) up-regulates Src kinase activity\[33\], and phosphorylation of a conserved tyrosine (Tyr527) in the carboxyl-terminal tail by Csk inactivates such activity\[34\]. To better understand the role of c-Src in the growth of leiomyoma cells, we further assayed the activity of c-Src in both cell types by detecting the level of phospho-Tyr416Src and phospho-Tyr527Src. We found that gossypol decreases the level of phospho-Tyr416Src and phospho-Tyr527Src. We found that gossypol decreases the level of phospho-Tyr416Src in both cell types, especially at the high concentration of 3.0 μmol/L. However, there was no significant alteration of the level of phospho-Tyr527Src after treatment with gossypol. The results indicate that gossypol reduces the activity of c-Src and has no impact on the inactivity of Src. Accordingly, we presumed that gossypol might be an active regulator of Src. Although gossypol was unable to directly reduce the expression of Src, decreasing the activity of Src may suppress the growth of uterine leiomyoma therapy.
myoma and induce apoptosis.

Moreover, because reducing the expression of ERα is a vital mechanism of action in the treatment of uterine leiomyoma, it is necessary to investigate the effect of gossypol on the expression of ERα. In this study, we showed that there is no pronounced difference in the expression of ERα in the two types of cells. These results agree with those previously reported by Jakimiuk et al and Strissel et al. We found that gossypol decreases the expression of ERα at the low concentration of 0.1 μmol/L but not at other concentrations; in addition, gossypol decreases the level of phospho-ser167ERα in a concentration-dependent manner. These observations suggest that gossypol down-regulates the activity of ERα. We hypothesize that this down-regulation may be correlated with the decreased Src activity. More studies need to be done.

Although gossypol has been extensively studied as an anti-proliferative agent in breast and prostate cancer, little is known about its relationship to the expression of ERα. This anti-proliferative agent in breast and prostate cancer, little is known about its relationship to the expression of ERα. This may be related to the data presented by Gilbert et al in 1995. They demonstrated that co-incubation of MCF-7 cells with gossypol (5 μmol/L) and estradiol (10 nmol/L) did not alter the effects of gossypol, and they therefore presumed that the anti-proliferative effects of gossypol were not influenced by the ER mechanism. However, Huang et al demonstrated that gossypol reduces the binding capacity to ER in normal endometrial cells at the concentration of 1 μmol/L. Wang et al presumed that gossypol exhibits a non-specific weak binding capacity to ER as a result of reducing protein synthesis. Accordingly, we speculated that, due to its weak binding capacity to ER, gossypol suppresses the expression of ERα only at a low concentration of 0.1 μmol/L and not at higher concentrations of 1.0 and 3.0 μmol/L.

We also noticed that pretreatment with PP2, the potent inhibitor of Src, elevated the level of ERα. There is an important feed-forward signaling loop involving estrogen, the ERα and Src. On the one hand, crosstalk between ERα and c-Src improve ERα transcriptional activity; on the other hand, the crosstalk also activates ERα proteolysis. Increased Src activity correlates with a shortened ERα half-life. Inhibition of cellular Src impairs ERα ubiquitilation and ERα loss, resulting in the accumulation of ERα. As a result, pretreatment with either PP2 or Src siRNA elevates the level of ERα. These results agree with those previously reported by Chu et al in breast cancer cells. Thus, we presume that another reason why pretreatment with gossypol could not down-regulate the level of ERα may be that high concentrations of gossypol reduce Src activity.

In summary, our findings provide evidence that gossypol inhibits the growth of leiomyoma cells and induces apoptosis. Unlike other hormone-based medicine used in clinical practice, gossypol reduces the level of ERα only at a low concentration. Besides the effect on Bcl-2, one of gossypol’s mechanisms of action may involve decreasing the activity of Src and ERα. These results may open new avenues for the therapy of uterine leiomyoma. The precise mechanism of action of gossypol remains to be elucidated, and more investigation is needed.

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Author contribution
Yan ZHU and Lin CAO designed the research; Shu-wu XIE, Jian-feng ZHANG, Ting-ting ZHANG, and Jie-yun ZHOU performed the research; Yang CAO analyzed the data; and Yan ZHU wrote the paper.

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