Gossypol inhibits the growth of MAT-LyLu prostate cancer cells by modulation of TGFβ/Akt signaling

JIAHUA JIANG¹, WEIPING YE² and YOUNG C. LIN²,³

¹Cancer Research Laboratory, Methodist Research Institute, Clarian Health Partners Inc., 1800 N. Capitol Avenue, Indianapolis, IN 46202; ²Laboratory of Reproductive and Molecular Endocrinology, College of Veterinary Medicine, ³The Ohio State University Comprehensive Cancer Center, The Ohio State University, 1900 Coffey Road, Columbus, OH 43210, USA

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Abstract. Gossypol (GP), a male contraceptive compound naturally present in cottonseed products, possesses antiproliferative and anti-metastatic effects in vitro and in vivo. However, the detailed mechanisms responsible for the effects of GP on the cell cycle of prostate cancer cells remain to be elucidated. In the present study, we investigated the effects of GP on the regulation of the cell cycle of rodent prostate cancer MAT-LyLu cells and the mechanisms of GP-induced growth inhibition. Our results showed that GP inhibited the cell proliferation and colony formation in a dose-dependent manner by the up-regulation of expression and secretion of transforming growth factor β1 (TGFβ1) and down-regulation of expression of Akt and phospho-Akt protein. The inhibition of cell growth was also demonstrated by cell cycle arrest at G0/G1 phase. Furthermore, GP decreased the expression of cyclin D₁, Cdk4 and phospho-Rb in MAT-LyLu cells. Thus, the inhibitory effects of GP on the proliferation of MAT-LyLu prostate cancer cells are associated with modulation of TGFβ1 and Akt signaling, which influence the expression of regulatory proteins such as cyclin D₁, Cdk4 and phospho-Rb which regulate cell cycle progression of prostate cancer cells.

Introduction

Prostate cancer is the most common malignancy and the second leading cause of male cancer-related death in the US, accounting for approximately 29% of all cancers diagnosed in males (1). The primary therapeutic strategies for the treatment of prostate cancer is androgen deprivation (2). Androgen withdrawal may initially reduce the growth of metastatic prostate cancers; however, the long-term treatment of prostate cancer patients results in loss of responsiveness. Prostate cancer cells finally become androgen independent, leading to the failure of androgen-deprivation therapy and patient death (2-4). Thus, it is necessary to look for new agents to treat androgen-independent prostate cancer.

Gossypol (GP) is a naturally occurring yellow pigment present in cottonseeds. It is well documented that GP inhibits the proliferation of many human cancer cells in vitro and in vivo (5-10). For example, GP significantly inhibited the growth of PC-3, DU-145, MCF-7 cells (6,8-10) as well as the in vivo growth and metastasis of prostate cancer MAT-LyLu cells after implantation into Copenhagen rats (5). GP induces cell cycle arrest at G0/G1 phase in human prostate cancer PC-3 cells (6) or induces apoptosis in PC-3 (11) and colon cancer HT-29 cells (12). (-)-GP, an enantiomer of GP, inhibits growth in breast cancer and prostate cancer cells as well as tumor growth of PC-3 cells in xenograft models (10,13-15).

Although the precise mechanism of action of GP remains unknown, it is believed that the anticancer effects of GP are related to apoptosis induction and cell cycle arrest at G0/G1 phase (6,11,12,14). The molecular mechanism of GP-induced apoptosis is well defined. It was reported that GP induced apoptosis by inhibiting the heterodimerization of anti-apoptotic proteins Bcl2/Bcl-xl with pro-apoptotic molecules Bax and Bim in human prostate cancer PC-3 cells (11) and colon cancer HT-29 cells (12). (-)-GP, an enantiomer of GP, inhibits apoptosis in breast cancer and prostate cancer cells as well as tumor growth of PC-3 cells in xenograft models (10,13-15).

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inhibition. TGFβ1 has been shown to mediate the anti-proliferative effects of many antitumor agents, including vitamin D₃, genistein, GP, and tamoxifen. TGFβ1 exerts its biological effects by binding to TGFβ receptor II (TβRII), which induces formation of a hetero-meric complex of TβRII and TβRI, triggering a signaling pathway that regulates cell cycle regulators such as Rb, cyclin and cyclin-dependent kinase (20,21). To date, the effect of GP on the cell cycle and TGFβ1 in MAT-LyLu cells remains unknown. Moreover, TGFβ1 has been recently reported to regulate the Akt signaling pathway in colon cancer cells, leading to inhibition of cell proliferation (22). Thus, we hypothesized that Akt may play an important role in GP-induced growth inhibition.

The objective of this study was to investigate the role of TGFβ1 and Akt signaling in GP-induced growth inhibition of MAT-LyLu prostate cancer cells.

Materials and methods

Reagents. GP was purchased from Sigma (St. Louis, MO). GP was dissolved in 100% ethanol to make a 10-mM stock solution. The solutions were aliquoted into glass vials and stored at -20°C. Treatment solutions were prepared by the dilution of stock solution in culture medium. Ultrapure natural human TGFβ1 was purchased from Genzyme Corp. (Cambridge, MA).

Cell culture. MAT-LyLu cells were the generous gift from P. Ghosh of Ohio State University, who originally obtained the cells through the courtesy of Dr J.T. Isaacs, Johns Hopkins University. Cells were cultured in RPMI-1640 medium (Invitrogen, Grand Island, NY) containing an antibiotic-antimycotic mixture (100 IU/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin) (Invitrogen, Carlsbad, CA) and 5% fetal calf serum (FCS; Atlanta Biologicals, Norcross, GA) in a humidified incubator (37°C, 5% CO₂ and 95% air).

Cell proliferation assay. Cell proliferation was determined by the tetrazolium salt (MTT) method according to the manufacturer's instructions (Promega, Madison, WI). MAT-LyLu cells (2500 cells per well) were cultured in a 96-well plate and were treated with different concentrations of GP (0.0-2.0 μM) for 24 h. At 24 h of incubation, the conditional media from cells were collected, and the secreted TGFβ1 protein was measured as previously described (9). The TGFβ1 concentration in the conditioned media was presented as pg/μg cell protein. The total cell protein in each well was harvested and determined as previously described (9).

Reverse transcription-polymerase chain reaction (RT-PCR). MAT-LyLu cells were treated with different concentrations of GP (0.0-2.0 μM) for 24 h. The total RNA isolation, complementary DNA (cDNA) synthesis and PCR for TGFβ1 and β-actin were performed as previously described (9). The final RT-PCR products (10 μl) were run on a 1.5% agarose gel containing ethidium bromide. The specific bands were quantified by ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Results were presented as the ratio of TGFβ1 to β-actin.

ELISA for TGFβ1 analysis. MAT-LyLu cells were cultured in 24-well culture plates and treated with GP (0.0-2.0 μM) for 24 h. At 24 h of incubation, the conditional media from cells were collected, and the secreted TGFβ1 protein was measured as previously described (9). The TGFβ1 concentration in the conditioned media was presented as pg/μg cell protein. The total cell protein in each well was harvested and determined as previously described (9).

Western blot analysis. MAT-LyLu cells were treated with different concentrations of GP (0.0-4.0 μM) for 24 h, and cell extracts were prepared as previously described (9). Equal amounts of proteins (20 μg/lane) were separated on 12% Tris-HCl gel (Bio-Rad Laboratories, Hercules, CA) and transferred to a PVDF membrane (Millipore, Bedford, MA). The membrane was incubated with the corresponding primary antibodies diluted 1:1000 in blocking solution, as indicated: a rabbit anti-phospho-Akt (ser473) polyclonal antibody (Cell Signaling Technology, Danvers, MA); a rabbit anti-Akt polyclonal antibody, a rabbit anti-cyclin D1 polyclonal antibody, a rabbit anti-ckd4 polyclonal antibody, a rabbit anti-p21 polyclonal antibody, a rabbit anti-phospho-Rb polyclonal antibody, a rabbit anti-Rb polyclonal antibody, and a mouse anti-β-actin monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), respectively. Anti-mouse or anti-rabbit secondary antibodies (Amersham Biosciences, Buckinghamshire, UK) were used for antibody recognition. Antibody-bound proteins were detected by ECL Western Blotting Detection System (Amersham Biosciences).

Statistical analysis. Data were expressed as the mean ± standard deviation (SD) of 4 culture wells. Statistical
differences between means were evaluated using one-way analysis of variance (ANOVA) followed by Tukey’s pairwise comparisons. A probability (p) <0.05 was considered significant.

Results

Effects of GP on the proliferation of MAT-LyLu cells. In order to evaluate whether GP possesses an anti-proliferative property in the androgen-independent prostate cancer cells, we examined the effects of GP on the growth of MAT-LyLu. MAT-LyLu cells were treated with increasing concentrations of GP (0.0, 0.5, 1.0, 2.0 and 4.0 μM) for 24, 48 and 72 h, and proliferation was determined by MTT assay. GP significantly inhibited the growth of MAT-LyLu cells as shown in Fig. 1A. At 24 h, GP at the concentrations of 1.0, 2.0 and 4.0 μM inhibited cell proliferation by 16.3, 28.8 and 43.4% respectively; at 48 h, GP at the concentrations of 1.0, 2.0 and 4.0 μM inhibited cell proliferation by 22.1, 37.3 and 67.5% respectively. The IC50 of GP for 48- and 72-h treatment was 2.64 and 1.25 μM, respectively. The results showed that GP inhibited the growth of MAT-LyLu cells in a dose- and time-dependent manner. In order to test whether GP has inhibitory effects on the metastatic potential of prostate cancer cells, we performed colony formation assay in GP-treated MAT-LyLu cells. GP at the same concentrations (0.0, 1.0, 20 and 4.0 μM) decreased the number of cell colonies (Fig. 1B). These results indicate that GP inhibits the anchorage-dependent (cell proliferation) and -independent growth (colony formation) of MAT-LyLu cells.

Effect of GP on the cell cycle distribution of MAT-LyLu cells. Since the inhibition of growth may be mediated by cell cycle growth arrest or apoptosis, we considered the possibility that the growth inhibition of MAT-LyLu cells induced by GP may be related to arrest at specific checkpoints in the cell cycle. To examine whether GP affects cell cycle distribution in MAT-LyLu cells, the cells were treated with different concentrations of GP (0.0, 1.0, 2.0 and 4.0 μM) for the indicated periods of time. Cell cycle distribution was performed by flow cytometry. As shown in Fig. 2, GP increased the percentage of the cell population in G0/G1 phase and decreased the percentage of the cell population in

![Figure 1](image1.png)
![Figure 2](image2.png)

Figure 1. Effects of gossypol (GP) on the proliferation of MAT-LyLu cells. (A) MAT-LyLu cells were treated with GP (0-4.0 μM). Cell proliferation was determined by the tetrazolium salt (MTT) method. (B) Anchorage-independent growth (colony formation) of MAT-LyLu cells was assessed on soft agarose containing GP (0-4.0 μM) after incubation for 14 days. Each bar represents the mean ± SD of 3 replicate samples. *Significantly different from the control group; p<0.05.

Figure 2. Effects of gossypol (GP) on the cell cycle distribution of MAT-LyLu cells. (A) MAT-LyLu cells were treated with GP (0-4 μM) for 24 h. The cell populations at G0/G1, S and G2/M phase were determined by flow cytometry. (B) MAT-LyLu cells were treated with GP (2.0 μM) for 0, 24 and 48 h. The cell populations at G0/G1, S and G2/M phase were determined by flow cytometry. Each bar represents the mean ± SD of 3 replicate samples. *Significantly different from the control group; p<0.05.
S phase. The percentage of cells in G0/G1 phase increased from 52.4 to 70.6%, while the percentage of cells in S phase decreased from 32.9 to 21.3% (Fig. 2A). Similarly, we also found that GP increased the percentage of cell population at G0/G1 phase in a time-dependent manner (Fig. 2B). These data showed that GP treatment resulted in cell cycle arrest of MAT-LyLu cells at G0/G1 phase in a dose- and time-dependent manner. Although GP at 1.0 and 2.0 μM caused an increase in the percentage of the cell population at G2/M phase, we did not observe this effect at a high concentration of GP (4.0 μM). Thus, the inhibitory effects of GP on MAT-LyLu cells may be due to cell cycle arrest at G0/G1 phase.

Effect of GP on the expression of cell cycle-regulated proteins in MAT-LyLu cells.

The progression of cell cycle is controlled by cyclin and cyclin-dependent kinase. In order to determine how GP modulates the expression of cell cycle regulatory proteins involved in the control of G1/S phase transition of the cell cycle, we investigated the expression of cyclin D1 and Cdk4 in MAT-LyLu cells by Western blot analysis (Fig. 3A). GP resulted in a decrease in Cdk4 and cyclin D1 protein expression in MAT-LyLu cells, but did not result in appreciable change in expression of p21 protein (data not shown). Similarly, GP also decreased the expression of phospho-retinoblastoma (Rb), but did not cause any significant change in the expression of Rb (Fig. 3B). Cancer cell growth arrest at G0/G1 phase by influencing the function of cyclin D1 and Cdk4 was also reported by other researchers (23,24). These data suggest that the G0/G1 phase cell growth arrest in MAT-LyLu cells by GP is mediated through the down-regulation of Cdk4 and cyclin D1, which further results in down-regulation of phosphorylation of Rb protein.

Effect of GP on the mRNA expression and secretion of TGFβ1 in MAT-LyLu cells.

The mechanism by which GP triggers an inhibitory pathway leading to growth inhibition of cultured
TGFß1, which resulted in G0/G1 cell cycle arrest of human prostate cancer PC-3 cells at G0/G1 phase of the cell cycle is known (6). However, how TGFß1 affects cell cycle-regulated proteins in GP-treated prostate cancer cells remains unknown. In this study, we examined the effects of GP on mRNA expression and secretion of TGFß1 in MAT-LyLu cells. RT-PCR results showed that GP at 1.0 and 2.0 μM resulted in a marked elevation of TGFß1 mRNA expression of MAT-LyLu cells, while the treatment of MAT-LyLu cells with 0.5 μM of GP had no significant effect on the expression level of TGFß1 mRNA (Fig. 4A). As expected, GP treatment significantly enhanced TGFß1 secretions of MAT-LyLu cells in a dose-dependent manner (p<0.05). GP at 0.5, 1.0 and 2.0 μM increased TGFß1 secretions by 1.05-, 1.38- and 1.43-fold compared to the control, respectively (Fig. 4B).

To confirm the ability of MAT-LyLu cells to respond to the inhibitory effects of exogenous TGFß1, the cells were treated with different concentrations of TGFß1 (0, 0.1, 1.0 and 10 ng/ml) for 24 h. Results showed that exogenous TGFß1 inhibited the growth of MAT-LyLu cells in a dose-dependent manner (Fig. 4C). TGFß1 (10 ng/ml) inhibited the DNA synthesis of MAT-LyLu cells by 63.8% compared to the control. Therefore, growth inhibition and G0/G1 phase cell cycle arrest in the GP-treated MAT-LyLu cells are mediated through the up-regulation of TGFß1 secretion.

Effect of GP on the expression of phospho-Akt and Akt in MAT-LyLu cells. Akt is a serine-threonine kinase which controls the activation of NF-κB, which regulates expression and activity of cyclin D1 in breast and prostate cancer cells (25-28). We investigated whether the inhibitory effects of GP are mediated through the suppression of Akt kinase in prostate cancer cells, which results in the reduction of cyclin D1 expression. The cell extracts from MAT-LyLu cells treated with GP (0-4.0 μM) were subjected to Western blot analysis. As seen in Fig. 5, GP resulted in the reduction in expression of Akt in MAT-LyLu cells in a dose-dependent manner. In addition, GP also markedly decreased phosphorylation of Akt at Ser473. Thus, cell cycle arrest at G0/G1 phase by GP is also the result of suppression of Akt activity, which results in down-regulation of cyclin D1 expression.

Discussion

We previously reported that GP up-regulated the expression of TGFß1, which resulted in G0/G1 cell cycle arrest of human prostate cancer and human BPH cells (6,9,29). In this study, we investigated the molecular mechanism of GP-induced cell cycle in rodent prostate cancer cells. We showed that GP inhibited the anchorage-dependent (cell proliferation) and anchorage-independent growth (colony formation) in MAT-LyLu cells. Consistent with its ability to inhibit cell proliferation of MAT-LyLu cells, GP inhibited colony formation, stimulated mRNA expression and secretion of TGFß1, and inhibited Akt activity of the MAT-LyLu cells in a dose-dependent manner. GP also inhibited the growth of prostate cancer cells by cell cycle arrest at G0/G1 phase. Although it has been reported that GP may arrest the cell cycle at S phase (30), our results revealed that GP might result in an accumulation of the cell population at G0/G1 phase of the cell cycle with a decrease in the cell population at S phase.

Our observation that GP induces cell cycle arrest at G0/G1 phase in MAT-LyLu cells is in accordance with our previous study demonstrating cell cycle arrest at G0/G1 phase in PC-3 (6) and human BPH cells (29). It is reasonable to assume that GP inhibits the growth of MAT-LyLu cells through cell cycle arrest at G0/G1 phase.

Since aberrantly active cell cycle regulatory proteins such as cyclin D1 and cyclin-dependent kinase (Cdk4) are responsible for the uncontrolled growth of cancer cells (31), these proteins are suitable therapeutic targets. Ligueros et al demonstrated that GP arrested the cell cycle at G1/S phase by decreasing Rb expression, Rb phosphorylation, and cyclin D1 protein expression in human MCF-7 breast cancer cells (8). Thus, we aimed to determine whether GP affects the expression of cell cycle-regulated proteins, such as Cdk4, cyclin D1, Rb and phospho-Rb in MAT-LyLu cells. We evaluated the influences of GP on the expression of cell cycle regulators such as cyclin D1, Cdk4 and Rb, which regulate cell cycle progression. As shown in Fig. 3A, GP markedly decreased cyclin D1 expression in a dose-dependent manner. Since cyclin D1 regulates the activity of cyclin-dependent kinase Cdk4, which controls the G1 cell cycle checkpoint, we investigated the effects of GP on Cdk4 kinase. As expected, GP also inhibited expression of Cdk4 suggesting that cell cycle arrest at G0/G1 phase by GP is a result of inhibition of cell cycle regulatory proteins such as cyclin D1 and Cdk4.

Since cyclin D1 forms a complex with Cdk4/Cdk6 to further phosphorylate Rb with the release of general transcription factor E2F1 which accelerates cell cycle progression from G1 to S phase (31), we subsequently examined the effects of GP on expression of phosphorylated Rb and total Rb in MAT-LyLu cells. GP suppressed the phosphorylation of Rb, but did not cause an obvious change in the level of expression of total Rb (Fig. 3B). Thus, the anti-proliferative effects were mediated through the down-regulation of expression of Cdk4 and cyclin D1, leading to the inhibition of phosphorylation of Rb and cell cycle arrest at G0/G1 phase. This observation is in agreement with our previous study demonstrating cell cycle arrest at G1/G0 phase of prostate cancers and down-regulation of cyclin D1 by GP (6,9). One clinical study showed that GP decreased cyclin D1 expression and increased the nuclear Rb expression in breast cancer patients (7). Huang et al showed that (-)-GP inhibited the growth of prostate cancer DU-145 cells through up-regulation of p21 and down-regulation of Cdk4, cyclin D1 and Rb.
proteins (10). Although we found inhibitory effects of GP on the expression of cyclin D1, Cdk4 and phospho-Rb in MAT-LyLu cells, we did not find any inhibitory effect of GP on the expression of total Rb and p21 (data not shown) in these cells.

TGFβ1 is a potent growth inhibitor in most epithelial cells including cancer cells. TGFβ1 mediates growth inhibition by blocking cell cycle progression from G1 to S phase. In most cases, it induces cell cycle arrest at G0/G1 phase via preventing the active cyclin D1-Cdk4 complex and Rb phosphorylation (21,32). TGFβ1 has been shown to decrease cyclin D1 expression in rat intestinal epithelial cells (33), expression of Rb and phosphorylated Rb in MvLu cells (34), and expression of c-Myc in keratinocytes (21). We previously showed that cell cycle arrest at G0/G1 phase of human prostate cancer and human BPH cells are mediated through stimulation of TGFβ1 expression (6,9,29). It is possible that inhibition of expression of cyclin D1 and Cdk4 by GP could be mediated by up-regulation of TGFβ1 secretion. In the present study, we demonstrated the stimulatory effects of GP on mRNA expression and secretion of TGFβ1 in MAT-LyLu cells. Moreover, the stimulatory effect of GP on secretion of TGFβ1 correlates well with its ability to arrest the cell cycle at G0/G1 phase. The results that MAT-LyLu cells secreted and responded to TGFβ1 suggest that TGFβ1 serves as a negative regulator for MAT-LyLu cells. These findings, along with the observations that GP reduced the expression of cyclin D1, Cdk4 and phospho-Rb protein of MAT-LyLu cells, suggest that the anti-proliferative activity of GP is mediated by inducing TGFβ1 protein production in MAT-LyLu cells, which further regulates the function of cyclin D1, Cdk4 and phospho-Rb protein in cell cycle progression. Our data suggest that GP probably exerts its effect at the transcriptional level within the cell cycle regulatory pathway.

Akt is a family of serine/threonine kinases and consists of three highly conserved kinases; Akt1, Akt2, Akt3. Elevated gene expression and kinase activities of Akt have been observed in many human cancers including prostate cancer (35), suggesting that Akt may play an important role in the development and progression of malignancy. Because Akt kinase controls the activation of NF-κB (25) and expression of NF-κB-regulated cyclin D1 (26), the suppression of cyclin D1 expression might be mediated by the inhibition of Akt kinase activity. A recent study showed that TGFβ1 treatment decreased phosphorylation of Akt at Ser473 in human colon cancer cells (22). This prompted us to hypothesize that GP may inhibit Akt phosphorylation directly or indirectly by modulating TGFβ1 signaling. In order to test this hypothesis, we examined the effects of GP on expression of Akt and phospho-Akt in MAT-LyLu cells. We demonstrated that GP suppresses the expression of Akt and activity of Akt by inhibiting phosphorylation of Akt at Ser473. Notably, isoflavone and genistein, which inhibit Akt phosphorylation, have been shown to inhibit expression of cyclin D1 in dorsolateral prostate tissues of TRAMP/FVB mice (36). Collectively, we conclude that GP induces cell cycle arrest at G0/G1 phase in MAT-LyLu cells by inhibition of Akt kinase activity, which suppresses expression of cyclin D1 and Cdk4 kinase leading to a decrease in the phosphorylation of Rb. Our results further confirm the role of Akt as an oncogene responsible for the growth and survival of cancer cells. Li et al demonstrated that the Akt pathway is a major survival pathway in human cancer, and the inhibition of Akt may suppress the activity of regulatory proteins of G1/S cell cycle progression, such as cyclin D1 and Rb (35).

In conclusion, we demonstrated that GP inhibited Akt, stimulated the expression and secretion of TGFβ1, and inhibited expression of cyclin D1 and phospho-Rb in prostate cancer cells. The inhibitory effects of GP on MAT-LyLu cells are mediated by up-regulating TGFβ1 secretion and down-regulating phosphorylation and expression of Akt kinase, which suppresses expression of cell cycle regulatory proteins cyclin D1, Cdk4 and phospho-Rb, leading to cell cycle arrest at G0/G1 phase and growth inhibition. Thus, GP exerts its effects on cancer cells by multiple mechanisms and may have potential therapeutic use for the treatment of prostate cancer.

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References