

Gossypol sensitizes the antitumor activity of 5-FU through down-regulation of thymidylate synthase in human colon carcinoma cells

Dan Yang^{1,3} · Jinglei Qu² · Xiujuan Qu² · Yubo Cao² · Ling Xu² · Kezuo Hou² · Wanyu Feng¹ · Yunpeng Liu²

Received: 2 February 2015 / Accepted: 14 April 2015 / Published online: 25 July 2015
© Springer-Verlag Berlin Heidelberg 2015

Abstract

Purpose 5-Fluorouracil (5-FU) is the basic chemotherapeutic agent used to treat colon cancer. However, the sensitivity of colon cancer cells to 5-FU is limited. Gossypol is a polyphenolic extract of cottonseeds. The purpose of this study was to investigate the activities and related mechanism of gossypol alone or in combination with 5-FU against human colon carcinoma cells.

Methods The IC₅₀ of gossypol or/and 5-FU in vitro was tested by 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, and the drug interaction was analyzed using the CalcuSyn method. Cell apoptosis was determined using presidium iodide staining and flow cytometric analysis. Western blotting was used to determine the expression of proteins. Transient transfection method was used to silence protein.

Results The IC₅₀ at 48 h of gossypol in colon cancer cells was 26.11 ± 1.04 μmol/L in HT-29 cells, 14.11 ± 1.08 μmol/L in HCT116 cells, and 21.83 ± 1.05 μmol/L in RKO cells. When gossypol was combined with 5-FU, a synergistic cytotoxic effect was

observed in HT-29 cells, HCT116 cells, and RKO cells compared with treatment with gossypol or 5-FU alone. The Western blotting results indicated that gossypol down-regulated thymidylate synthase (TS) rather than thymidine phosphorylase protein expression. Furthermore, the mTOR/p70S6K1 signaling pathway was inhibited in gossypol-treated colon cancer cells, and consequently, cyclin D1 expression was decreased, suggesting an additional mechanism of the observed antiproliferative synergistic interactions. All the observation was confirmed by silencing TS and inactivating the mTOR/p70S6K1 signaling pathway by rapamycin, both of which increased the chemo-sensitizing efficacy of 5-FU.

Conclusions These findings suggest that gossypol-mediated down-regulation of TS, cyclin D1, and the mTOR/p70S6K1 signaling pathways enhances the anti-tumor effect of 5-FU. Ultimately, our data exposed a new action for gossypol as an enhancer of 5-FU-induced cell growth suppression.

Keywords Colon cancer · Gossypol · 5-Fluorouracil · Thymidylate synthase · Cyclin D1 · mTOR

✉ Wanyu Feng
ss5632fengyu@126.com

✉ Yunpeng Liu
syliuyang123625@163.com

¹ Department of Pharmacology, China Medical University, No. 77 Puhe Road, Shenyang North New Area, Shenyang City 110122, People's Republic of China

² Department of Medical Oncology, The First Hospital, China Medical University, No. 155, North Nanjing Street, Heping District, Shenyang City 110001, People's Republic of China

³ Department of Pharmacology, Shenyang Medical College, No. 146, North Huanghe Street, Huanggu District, Shenyang City 110034, People's Republic of China

Introduction

Colorectal cancer (CRC), with approximately 1 million new cancer cases each year, is the fourth leading cause of cancer-related deaths worldwide [1, 2]. 5-Fluorouracil (5-FU) is one of the most important pharmacological agents in the treatment of colon cancer [3, 4]. In the past few decades, 5-FU and 5-FU-based chemotherapy were mainstream in the adjuvant treatment of CRC [5, 6]. However, the gains made by the chemotherapeutic efficacy of 5-FU and 5-FU-based chemotherapy are somewhat limited

in patients with colon cancer, primarily due to the development of drug resistance [7–9]. Therefore, there is an urgent need to develop new therapeutic strategies to enhance the anti-tumor effect of 5-FU.

Gossypol, a polyphenolic compound, is naturally present in cottonseed products. Initially, it was only used as a male fertility-control agent but is now widely used. The anticancer properties of gossypol have been studied in a variety of cancers since the 1980s [10–14]. Treatment with gossypol may have various biochemical and molecular impacts on different cancers with specific biological behaviors. Gossypol is proposed to inhibit Bcl-2 and Bcl-XL and can also induce autophagic cell death as well as apoptosis through the Smac, p53, and caspase pathways [15–17]. Another study also showed that gossypol caused cancer cell cycle arrest at the G2/M or G0/G1 phases along with the down-regulation of Akt and phospho-Akt protein expression and the decreased expression of cyclin D1, Cdk4, and phospho-Rb due to the up-regulation of TGF-beta1 expression and secretion [18, 19].

In addition, gossypol has been shown to effectively enhance anticancer agent-induced apoptosis in combination with other conventional chemotherapeutic drugs. Gossypol can overcome gemcitabine resistance in cell lines with a high level of Bcl-2 expression and sensitize cancer cells to TRAIL or docetaxel in combination drug therapy [20–22]. Currently, gossypol is being evaluated in phase I and II clinical trials for use as a single agent or in combination with other anti-tumor agents in a variety of hematologic, lymphoid, and solid tumor malignancies [23, 24]. However, the potential of gossypol to improve 5-FU sensitivity of colon cancer cells is unclear.

TS is an enzyme involved in DNA synthesis and a target of 5-FU [25]. However, studies indicate that 5-FU treatment induces TS expression, which might lead to chemoresistance [26, 27]. The low level of TS is generally thought to sensitize cells to 5-FU. Thymidine phosphorylase (TP) is a key enzyme that contributes in the metabolic process of pyrimidine nucleotides [28]. TP expression can contribute to predicting sensitivity of colorectal cancer cells to 5-FU [29], and the patients with high TP expression and treated with 5-FU exhibited better prognosis [30].

The mTOR kinase is a key element in intracellular signal transduction that is responsible for the regulation of cell growth and proliferation [31]. mTOR kinase can phosphorylate and activate p70S6K1, which is a key mediator of mTOR function and also can regulate diverse cellular processes including protein synthesis, cell growth, and survival [32–34]. Inhibiting phosphorylated p70S6K1 suppresses the proliferation and growth of carcinoma and overcomes the adaptive resistance to targeted therapies [35–37]. Studies also showed that the inhibition of the mTOR/p70S6K1 signal may be a key molecular event in enhancing 5-FU-induced apoptosis [38].

Fig. 1 Effects of 5-FU or gossypol on HT-29 cells, HCT116 cells, and RKO cells. **a** Cell proliferation of HT-29 cells, HCT116 cells, and RKO cells incubated with the indicated concentrations of gossypol (for 24 or 48 h). Cell growth inhibition was assessed by the MTT assay. *Points* represent the mean \pm SD. Sigmoidal dose response curves were derived from fitting the data to a nonlinear regression program (Graph Pad Prism). **b** The percentage of apoptotic HT-29 cells, HCT116 cells, and RKO cells incubated with the indicated concentrations of gossypol for 48 h. Apoptosis (APO) was analyzed as a sub-G1 fraction by flow cytometry with PI staining. **c** Western blotting showed that gossypol induced procaspase-3 and PARP cleavage in HT-29 cells, HCT116 cells, and RKO cells. **b, c** The final results are summarized in the bar graphs. Data are presented as the mean \pm SD of three independent experiments. * $p < 0.01$ compared to the cells in the control group

In the present study, we investigated the ability of the combination of gossypol and 5-FU to sensitize human colon cancer cells to 5-FU-induced apoptosis. Our data show that gossypol sensitized colon cancer cells to 5-FU via the induction of apoptosis through the down-regulation of TS expression, which is partially regulated by the down-regulation of the mTOR/p70S6K1 signaling axis.

Materials and methods

Reagents and antibodies

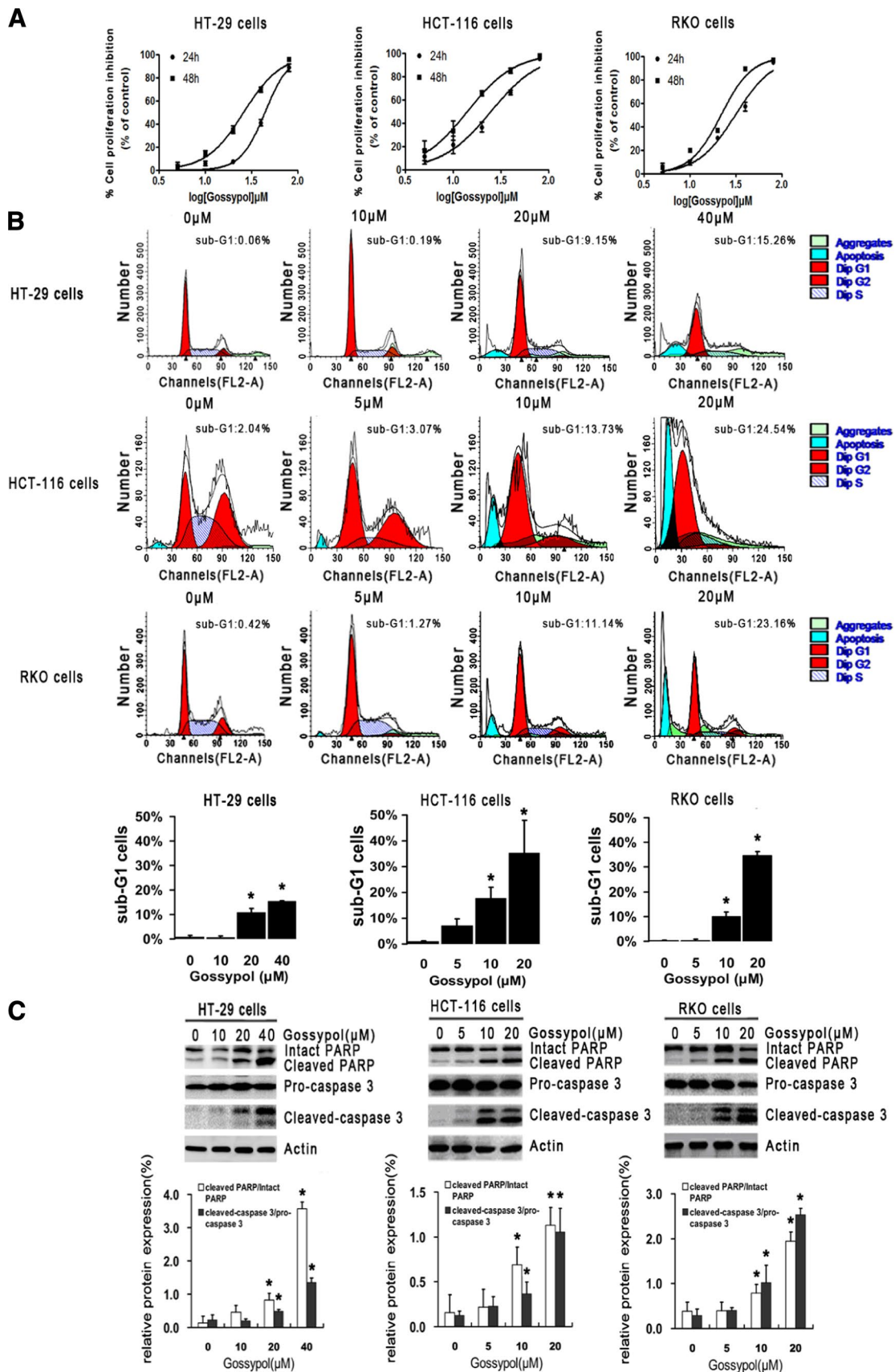
Gossypol (Sigma-Aldrich; St Louis, MO, USA) was dissolved in DMSO at a concentration of 20 mM and was stored at -20°C . Anti-cyclin D1, anti-actin, anti-TS, and anti-TP antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-poly (ADP-ribose) polymerase (PARP), anti-phospho-mTOR, anti-mTOR, anti-phospho-p70S6K1, anti-p70S6K1, and anti-caspase-3 antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA).

Cell culture

The cells were cultured in RPMI 1640 medium (Gibco) containing 10 % heat-inactivated fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 mg/mL) at 37°C under an atmosphere of 95 % air and 5 % CO_2 . Cells were routinely subcultured every 2–3 days, and all of cell samples were in the logarithmic growth phase.

Cell proliferation assay

The effects of 5-FU and/or gossypol on cell proliferation were measured using the 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The cells were seeded at 5×10^3 cells/well in 96-well plates and incubated overnight, and then, different concentrations of



5-FU and/or gossypol were added and further incubated for the indicated times. Thereafter, 20 μL of MTT solution (5 mg/mL) was added to each well, and the cells were incubated for another 4 h at 37 °C. After removal of the culture medium, the cells were lysed in 200 μL of dimethylsulfoxide, and the optical density (OD) was measured at 570 nm with a microplate reader (Model 550, Bio-Rad Laboratories, USA). The following formula was used: cell proliferation inhibition rate = $(1 - \text{OD of the experimental sample} / \text{OD of the control group}) \times 100\%$. Drug interaction was evaluated by using CalcuSyn software (version 2; Biosoft). Drug combination studies were analyzed with the combination index (CI); CI values <1.00 were considered as showing synergetic effects.

Flow cytometry analysis

The cells were seeded at 1×10^5 cells/well in six-well plates and were incubated overnight and then exposed to 5-FU and/or gossypol for 48 h. The cells were collected and washed twice with phosphate-buffered saline (PBS). After fixing in ice-cold 70 % ethanol for 12 h, the samples were washed twice with PBS and then incubated with 20 $\mu\text{g}/\text{mL}$ RNase A and 10 $\mu\text{g}/\text{mL}$ propidium iodide (PI) for 30 min in the dark. Finally, the samples were evaluated by flow cytometry, and the data were analyzed with ModFit 2.0 software.

Western blotting analysis

Cells were washed twice with PBS buffer and solubilized in 1 % Triton lysis buffer on ice. The protein concentration was determined using the Lowry method. Total proteins (30–50 μg) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (Immoblin-P, Millipore, Bedford, MA, USA). The membranes were blocked with 5 % skim milk in TBST buffer and then incubated with the indicated antibodies at 4 °C overnight. After washing with TBST, the membrane was reacted with horseradish peroxidase-conjugated secondary antibodies for 30 min at room temperature. The immunoreactive proteins were visualized with chemiluminescence reagent (SuperSignal Western PicoChemiluminescent Substrate, Pierce, USA).

Transient transfection

The cells were transiently transfected with control siRNA or TS siRNA using Lipofectamine 2000 reagent according to the manufacturer's protocol (Invitrogen, Life Technologies, Grand Island, NY, USA). Briefly, the diluted DNA sample as well as the transfection reagent were mixed at a 1:1 ratio and were added to 60–70 % confluent cells

and incubated for 5–7 h. The reaction was then stopped by replacing the transfection media with normal growth media.

For transfection, cells were seeded into six-well plates (1×10^5 cells/well) without antibiotics. After 24 h, the cells were transfected with TS siRNAs using Lipofectamine 2000 reagent (Invitrogen, USA) according to the manufacturer's instructions. After 48 h of transfection, the cells were subcultured for further use.

Statistical analysis

All experiments were repeated at least three times. Data are expressed as the mean \pm SD unless noted otherwise. Differences between data groups were evaluated for significance using Student *t* test. *p* values <0.01 were considered statistically significant.

Results

Growth inhibition of HT-29, HCT116, and RKO cells by gossypol

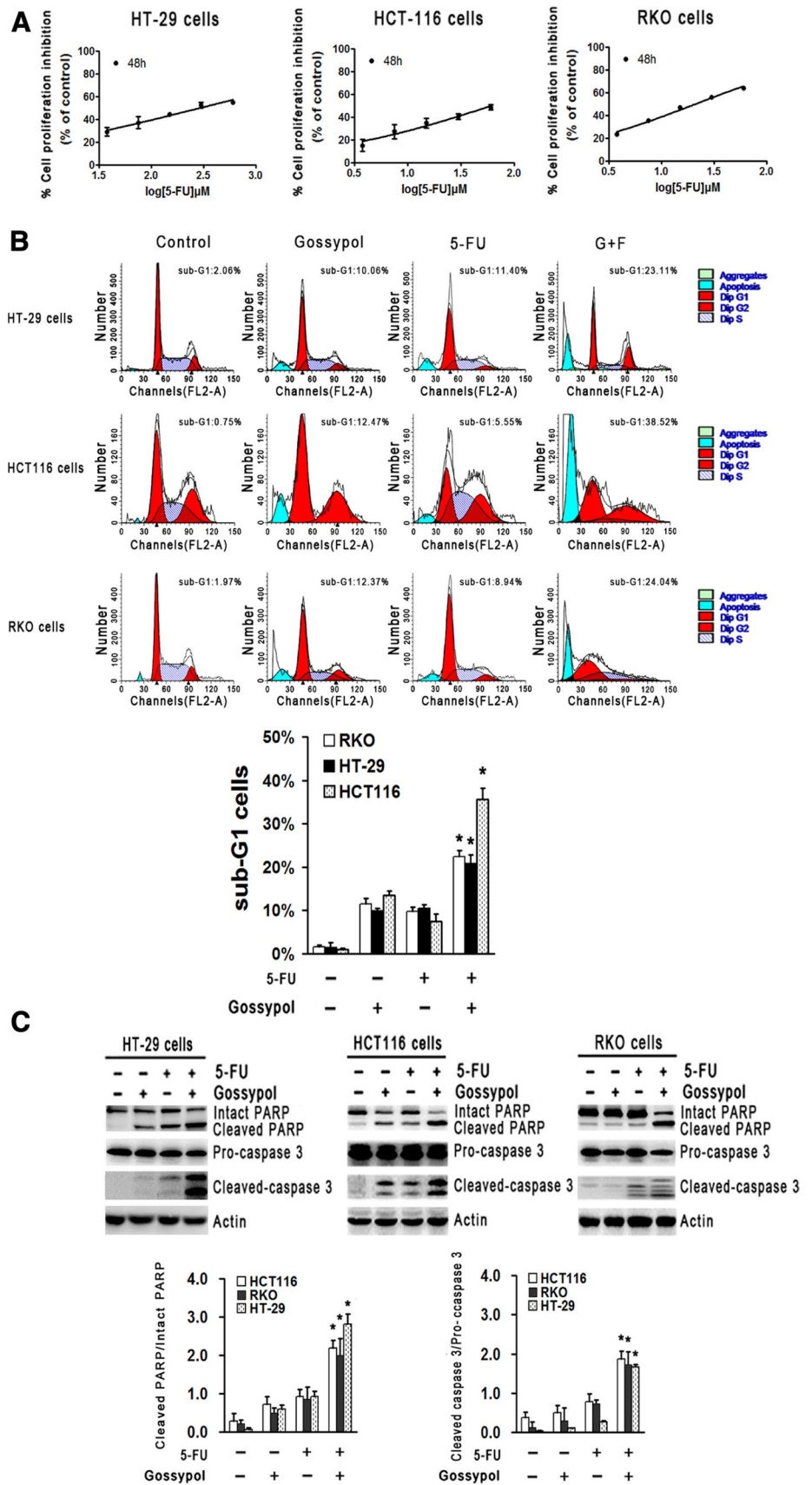
We tested the effects of gossypol on colon cancer cell lines. The effect of gossypol on the proliferation of HT-29, HCT116, and RKO cells was examined by the MTT assay (Fig. 1a). The results demonstrated that gossypol induced an anti-proliferative effect on colon cell lines in a time- and dose-dependent manner, and the IC_{50} at 48 h was 26.11 ± 1.04 $\mu\text{mol}/\text{L}$ in HT-29 cells, 14.11 ± 1.08 $\mu\text{mol}/\text{L}$ in HCT116 cells, and 21.83 ± 1.05 $\mu\text{mol}/\text{L}$ in RKO cells.

To understand how gossypol inhibits cell proliferation, the effect of gossypol on cell apoptosis was assessed. We found that treatment with gossypol for 48 h induced colon cells apoptosis in a concentration-dependent manner (Fig. 1b). When gossypol was used after 24 h, the cleavage of PARP (the downstream target of caspase-3) and procaspase-3 was detected (Fig. 1c). These results indicated that gossypol induced apoptosis in colon cancer cells.

Gossypol enhances 5-fluorouracil-induced apoptosis in colon cells

HT-29, HCT116, and RKO cells were treated with increasing concentrations of 5-FU, and the IC_{50} at 48 h was 293.70 ± 2.04 $\mu\text{mol}/\text{mL}$ in HT-29 cells, 61.48 ± 2.24 $\mu\text{mol}/\text{mL}$ in HCT116 cells, and 21.16 ± 1.82 $\mu\text{mol}/\text{mL}$ in RKO cells (Fig. 2a). The effect of gossypol in combination with 5-FU was then investigated. The combination of gossypol and 5-FU was found to induce a synergistic cytotoxic effect compared with that caused by either of the two compounds alone in colon

Fig. 2 Gossypol increased chemotherapeutic sensitivity against colon cancer in combination with 5-FU. **a** Cell proliferation of HT-29 cells, HCT116 cells, and RKO cells incubated with the indicated concentrations of 5-FU (for 48 h). Cell growth inhibition was assessed by MTT assay. *Points* represent the mean \pm SD. The sigmoidal dose–response curves were derived from fitting the data to a nonlinear regression program (GraphPad Prism). **b** The percentage of apoptotic cells was quantitated by flow cytometry after PI staining (150 μ mol/mL 5-FU and 20 μ mol/L gossypol in HT-29 cells, 30 μ mol/mL 5-FU and 10 μ mol/L gossypol in HCT116 cells and 15 μ mol/mL 5-FU and 20 μ mol/L gossypol in RKO cells for 48 h). **c** Western blotting showed gossypol combined with 5-FU-induced procaspase-3 and PARP cleavage in HCT116 cells and RKO cells (150 μ mol/mL 5-FU and 20 μ mol/L gossypol in HT-29 cells, 30 μ mol/mL 5-FU and 10 μ mol/L gossypol in HCT116 cells and 15 μ mol/mL 5-FU and 10 μ mol/L gossypol in RKO cells for 24 h). **b**, **c** The final results are summarized in the *bar graphs*. Data are presented as the mean \pm SD of three independent experiments. * p < 0.01 compared to the cells in the 5-FU treated group



cancer cells. The CI value was <1 in all colon cancer cells for the combination, indicating synergism (Table 1). As shown in Fig. 2b, the combination of 150 $\mu\text{mol}/\text{mL}$ 5-FU and 20 $\mu\text{mol}/\text{L}$ gossypol in HT-29 cells, 30 $\mu\text{mol}/\text{mL}$ 5-FU and 10 $\mu\text{mol}/\text{L}$ gossypol in HCT116 cells, and 15 $\mu\text{mol}/\text{mL}$ 5-FU and 20 $\mu\text{mol}/\text{L}$ gossypol in RKO cells for 48 h induced a significant enhancement of apoptotic cells, which was quantitated as a percentage by flow cytometry and through the significant cleavage of procaspase-3 and PARP to its active fragments in comparison with 5-FU and gossypol alone (Fig. 2c). The results indicate that gossypol enhances the cytotoxicity and apoptosis induced of 5-FU in human colon cancer cells.

Gossypol increased chemotherapeutic sensitivity against colon cell lines in combination with 5-FU through the down-regulated expression of TS, but not TP

To evaluate the role of gossypol in the regulation of the protein levels of TS in colon cell lines, HCT116 and RKO cells were exposed to various concentrations of gossypol for 24 h, and the protein levels of TS were determined by Western blot analysis. The results showed that gossypol significantly decreased the protein levels of TS in a dose-dependent manner (Fig. 3a). Next, we examined the combined effects of treatment with gossypol and 5-FU on the expression of TS. As shown in Fig. 3b, gossypol significantly down-regulated the 5-FU-induced up-regulation of TS levels and a clear ternary complex between FdUMP, TS and the methyl donor, as shown by the appearance of the upper 38 kDa band. Notably, the formation of the ternary complex is always achieved after combined treatment, indicating that gossypol does not affect the biochemical inhibition of TS induced by 5-FU. We then checked whether the down-regulation of TS by gossypol has any regulatory role in the enhancement effects of gossypol with 5-FU in colon cancer cells. Hence, we silenced TS expression by transiently transfecting the HCT116 cells with TS siRNA, and the proliferation of these cells treated with gossypol and/or 5-FU was compared to cells transfected with control siRNA. The results showed that the silencing of TS could

Fig. 3 Gossypol increased chemotherapeutic sensitivity against colon cell lines in combination with 5-fluorouracil through the down-regulated expression of TS proteins. **a** Gossypol induces the down-regulation of TS protein expression in HCT116 cells and RKO cells. **b** The effect of gossypol on 5-FU-induced activation of TS in HCT116 cells and RKO cells. **c** Small interfering RNA (siRNA)-mediated silencing of TS expression in HCT116 cells. Cells were transiently transfected with control and TS siRNA. **d** The effect of 5-FU and gossypol, alone or in combination, on control and TS siRNA-transfected HCT116 cells. The percentage of apoptotic cells was quantitated by flow cytometry after staining. Western blotting showed gossypol combined with 5-FU-induced PARP cleavage in HCT116 cells. **a, b, c** The expression of TS and TP proteins were analyzed by Western blotting. The final results are summarized in the bar graphs. Data are presented as the mean \pm SD of three independent experiments. **a, b** $*p < 0.01$ compared to the cells in the 5-FU treated group. **c, d** $*p < 0.01$ compared to the cells in the control siRNA-treated group

enhance the cytotoxicity of 5-FU and the combination of gossypol and 5-FU in HCT116 cells, indicating that gossypol treatment increased 5-FU-induced apoptosis, at least in part, through the down-regulation of TS.

In addition, we also analyzed the effect of gossypol in regulating the protein levels of TP. As shown in Fig. 3a, b, the results showed that the expression of TP was not obviously altered when treated with gossypol alone or in combination with 5-FU.

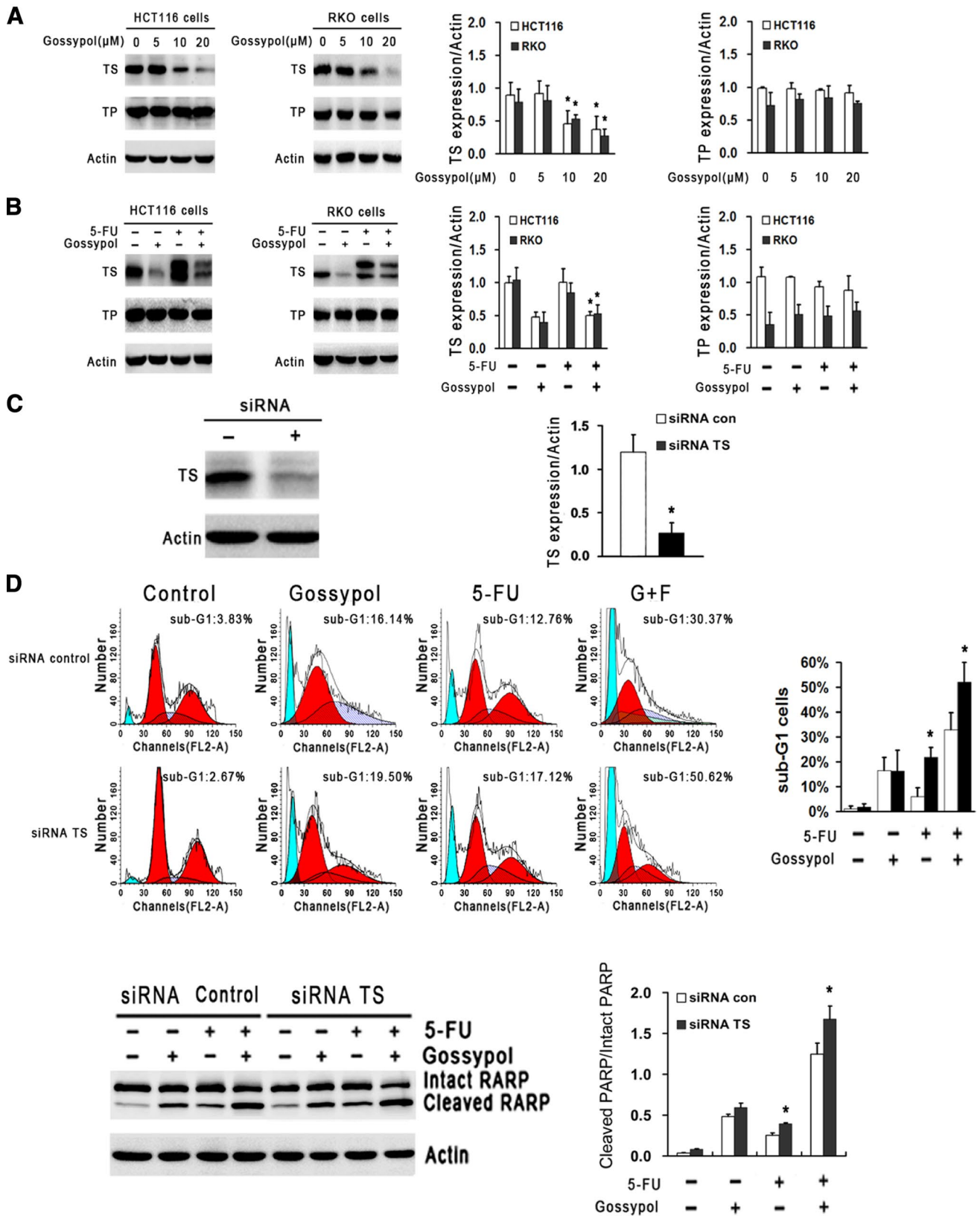
Gossypol treatment down-regulated the expression of TS partially through the down-regulation of the mTOR signaling pathway in colon cell lines

Our data suggested that treating HCT116 cells and RKO cells with gossypol leads to a dose-dependent reduction in the activated form of mTOR and p70S6K1, and down-regulation of cyclin D1 protein levels (Fig. 4a). Next, we examined the combined effects of co-treatment with gossypol and 5-FU on the levels of phospho-mTOR, phospho-p70S6K1, and cyclin D1. As shown in Fig. 4b, the levels of phospho-mTOR, phospho-p70S6K1, and cyclin D1 were also down-regulated. These data indicated that gossypol down-regulated the mTOR/p70S6K1 signal axis, which might take part in the down-regulation of TS expression. Our next effort was to check whether rapamycin (a

Table 1 Combination index values for the combination of gossypol ($\mu\text{mol}/\text{L}$) with 5-FU ($\mu\text{mol}/\text{L}$) in a panel of colon cancer cell lines

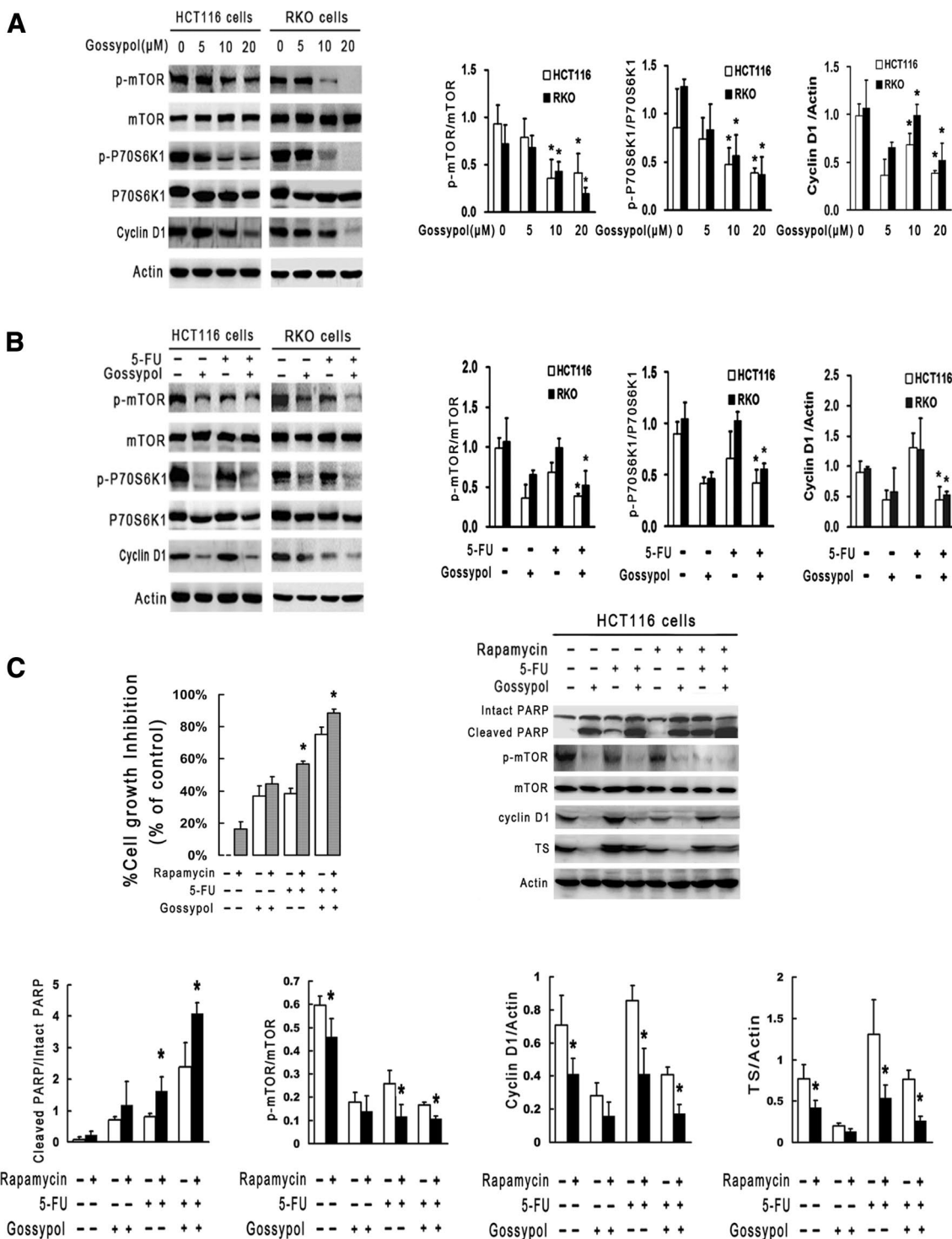
Fa	HCT116		RKO		HT-29	
	Gossypol/5-FU	CI	Gossypol/5-FU	CI	Gossypol/5-FU	CI
0.50	4.883/14.650	0.569 ± 0.041	7.889/5.917	0.459 ± 0.049	9.717/72.879	0.658 ± 0.132
0.60	6.027/18.080	0.33 ± 0.043	9.869/7.402	0.365 ± 0.041	12.428/93.212	0.557 ± 0.102
0.70	7.579/22.737	0.206 ± 0.044	12.598/9.448	0.295 ± 0.037	16.250/121.878	0.527 ± 0.092
0.80	10.024/30.073	0.129 ± 0.043	16.988/12.725	0.238 ± 0.037	22.539/169.039	0.542 ± 0.095
0.90	15.268/45.803	0.072 ± 0.037	26.554/19.916	0.190 ± 0.039	36.869/276.516	0.607 ± 0.126

Note: The CalcuSyn analysis included data from three separate experiments



classic mTOR inhibitor) might increase the cytotoxicity of 5-FU and decrease the protein levels of cyclin D1 and TS as efficiently as gossypol in colon cell lines. HCT116 cells

were exposed to 100 nmol/L rapamycin co-treatment with gossypol/5-FU as single or in combination. Cell proliferation was measured with MTT assay, and the expressions



of cleaved PARP, p-mTOR, mTOR, cyclin D1, and TS proteins were analyzed by Western blot. The results demonstrated that, as seen with gossypol treatment, rapamycin treatment also significantly increased the cytotoxicity of 5-FU and inhibited the mTOR/p70S6K1 pathway and decreased the expression of cyclin D1 and TS proteins (Fig. 4c). These data confirmed that gossypol down-regulated the expression of TS and cyclin D1, potentially

through the partial down-regulation of the mTOR/p70S6K1 signaling pathway in colon cell lines.

Discussion

5-FU is a standard chemotherapeutic drug that has been widely used as a standard chemotherapy drug in colorectal

Fig. 4 Gossypol treatment down-regulated the expression of TS and cyclin D1 partially through the down-regulation of the mTOR signaling pathway in colon cell lines. **a** Gossypol induces the down-regulation of phospho-mTOR, phospho-p70S6K1, and cyclin D1 protein expression in HCT116 cells and RKO cells. The cells were treated with various concentrations of gossypol for 24 h ($*p < 0.01$ compared to the cells in the control group). **b** The effect of 5-FU and gossypol, alone or in combination, on the levels of phospho-mTOR, phospho-p70S6K1, and cyclin D1 in HCT116 cells and RKO cells. The cells were treated with gossypol or/and 5-FU for 24 h ($*p < 0.01$ compared to the cells in the 5-FU treated group). **c** The effect of rapamycin treatment on the levels of phospho-mTOR, phospho-p70S6K1, TS, and cyclin D1 in HCT116 cells and RKO cells. The cells were treated with 100 nm rapamycin for 24 h. Cell proliferation of HCT116 cells incubated with 30 $\mu\text{mol/L}$ 5-FU and 10 $\mu\text{mol/L}$ gossypol, alone or in combination, with or without 100 nmol/L rapamycin. Cell growth inhibition was assessed by the MTT assay. *Points* represent the mean \pm SD ($*p < 0.01$ compared to the cells in the control group). Western blotting showed gossypol combined with 5-FU-induced PARP cleavage in HCT116 cells. **d** The effect of 5-FU and gossypol, alone or in combination, with or without rapamycin on the levels of cleaved PARP, phospho-mTOR, cyclin D1, and TS in HCT116 cells ($*p < 0.01$ compared to the cells in the 5-FU treated group). **a, b, c, d** The levels of cleaved PARP, phospho-mTOR, phospho-p70S6K1, mTOR, p70S6K1, TS, and cyclin D1 were detected by Western blotting

cancer. However, primary or acquired 5-FU resistance frequently develops and is a main cause of chemotherapy failure in human colorectal cancer [39]. It is therefore urgent to unravel the mechanisms underlying drug resistance in colorectal cancer as well as to finding new drugs that could enhance the susceptibility of cancer cells to 5-FU. In this study, the results demonstrated that gossypol exerted potent anti-proliferative effects in human colon cell lines. Moreover, combined treatment with 5-FU and gossypol showed a clear synergistic anti-proliferative effect and induced apoptosis. These studies suggested that co-treatment with gossypol may improve the efficiency of 5-FU in colon cancer therapy. To study the mechanism that how gossypol increased the chemo-sensitizing efficacy of 5-FU, in the following study, we used HCT116 cell line and RKO cell line as examples.

TS is a critical target of 5-FU, which catalyses the methylation of dUMP to dTMP. dTMP is then intracellularly metabolized to dTTP, an essential precursor for DNA biosynthesis [40, 41]. Previous studies in cancer patients have demonstrated that TS is the main molecular mechanism governing 5-FU sensitivity, and the over-expression of TS has been reported to be the mechanisms for the chemoresistance to 5-FU [42, 43]. A meta-analysis study showed that colorectal cancer patients with enhanced TS expression show a decrease in overall survival compared with those patients with low TS expression [44]. Furthermore, several clinical studies showed that the degree of inhibition of TS and the persistence of inhibition are essential factors for maximal in vivo growth inhibition by 5-FU [40, 45].

Multiple in vitro investigations have shown an improved response to 5-FU with low level of TS protein and enzyme activity in cancer cells [26, 46–48]. The HDAC inhibitor vorinostat had been shown to enhance the efficacy of 5-FU through down-regulation of TS [47]. Sinomenine was also shown to have the ability to reduce the expression of TS mRNA and sensitize gastric cancer cells to 5-FU in vitro and in vivo [45]. TP is a key enzyme that is able to catalyze the conversion of 5-FU to its active cytotoxic form, 5-fluoro-2'-deoxyuridine (5-FdUR) [49]. Activated 5-FdUR can inhibit TS activity, thereby depriving the de novo dTTP supply and blocking the process of DNA synthesis [50, 51]. Therefore, high TP expression in the tumor could theoretically promote the cytotoxicity of 5-FU [52].

We observed that gossypol could down-regulate TS protein expression and act to inhibit TS induction by 5-FU in co-treated colon cancer cell lines. However, gossypol did not affect the formation of the stable and inactive ternary complex between the 5-FU-metabolite FdUMP (5-fluoro-2'-deoxyuridine 5'-monophosphate), TS, and the methyl donor CH₂THF, indicating that it does not influence the biochemical inhibition of TS. In addition, we demonstrated that the addition of TS siRNA could further enhance the effectiveness 5-FU treatment alone or in combination with gossypol. Taken together, our data show, for the first time, that TS down-regulation is induced by gossypol in colon cancer cells treated with 5-FU. In addition, we demonstrated that the treatment of two colon cancer cell lines with gossypol did not alter TP expression levels and that TP was not involved in the enhanced effect of 5-FU when combined with gossypol in colon carcinoma.

The TS gene is one of the target genes of the transcription factor E2F1 [53]. In normal cell cycle progression, E2F exerts a repressive effect on E2F-responsive genes in the G₀/G₁ phase by associating with the retinoblastoma tumor suppressor gene product pRb and the related protein p130. This repression is relieved by the phosphorylation of the pRb family of proteins by the formation of a G₁ cyclin (cyclin D and cyclin E)–CDKs (cyclin-dependent kinases) complex, resulting in the expression of E2F-responsive genes in late G₁ with a peak at the G₁/S boundary [54]. Researchers have also reported that the suppression of cyclin D1 expression leads to decreased TS protein levels [55–57]. In the present study, we found that treatment with gossypol alone and in combination with 5-FU clearly decreased the expression level of cyclin D1, confirming that gossypol down-regulated TS expression through the inhibition of cyclin D1 expression.

Other researchers have reported that the mTOR inhibitors could synergize with 5-FU by inhibiting E2F1, TS, and cyclin D1 and by enhancing DNA damage in gastric cancer cells, colorectal cancer cells, and breast cancer cells

[58–66]. Thus, we hypothesized that the mTOR kinase may be regulated by gossypol.

Our data showed that treatment of gossypol led to a dose-dependent reduction in phospho-mTOR and phospho-p70S6K1 in colon cancer cell lines. Co-treatment with gossypol and 5-FU also down-regulated the expression levels of phospho-mTOR and phospho-p70S6K1. These results were similar to those of a study by Cheng et al. [67], which showed that apogossypolone (one of gossypol derivatives) negatively regulated the phosphorylation of mTOR in human hepatocellular cells. Furthermore, we demonstrated that the classic mTOR inhibitor rapamycin inhibited the mTOR/p70S6K1 pathways, down-regulating the expression of the cyclin D1 protein and TS. Thus in the present study, we confirmed that similar to rapamycin, the down-regulation of cyclin D1 and TS by gossypol alone or in combination with 5-FU occurred at least partially through the down-regulation of phospho-mTOR and phospho-p70S6K1.

Conclusions

In summary, the present results demonstrated that gossypol mediated the down-regulation of TS and the cyclin D1 protein and the inhibition of the mTOR/p70S6K1 signaling pathway to enhance the anti-tumor effect of 5-FU. These findings provide a rationale for the design of a novel combination of chemotherapy regimens for patients with colon cancer.

Acknowledgments This study was supported by the Chinese National Foundation of National Sciences grants (No. 81372546), Specialized Research Fund of Doctoral Programme of Higher Education (20112104110005), Science and Technology Plan Project of Liaoning Province (Nos. 2011404013-1, 2012225001).

Conflict of interest The authors declare no conflict of interest.

References

- Likui W, Hong W, Shuwen Z, Yuangang Y, Yan W (2011) The potential of osteopontin as a therapeutic target for human colorectal cancer. *J Gastrointest Surg* 15(4):652–659
- Antonic V, Stojadinovic A, Kester KE, Weina PJ, Brucher BL et al (2013) Significance of infectious agents in colorectal cancer development. *J Cancer* 4(3):227–240
- Chen J, Lu H, Yan D, Cui F, Wang X, Yu F et al (2015) PAK6 increase chemoresistance and is a prognostic marker for stage II and III colon cancer patients undergoing 5-FU based chemotherapy. *Oncotarget* 6(1):355–367
- de la Cruz-Morcillo MA, Valero ML, Callejas-Valera JL, Arias-Gonzalez L, Melgar-Rojas P, Galan-Moya EM et al (2012) P38MAPK is a major determinant of the balance between apoptosis and autophagy triggered by 5-fluorouracil: implication in resistance. *Oncogene* 31(9):1073–1085
- Van Loon K, Venook AP (2011) Adjuvant treatment of colon cancer: what is next? *Curr Opin Oncol* 23(4):403–409
- Soreide K, Berg M, Skudal BS, Nedreboe BS (2011) Advances in the understanding and treatment of colorectal cancer. *Discov Med* 12(66):393–404
- Shakibaei M, Mobasheri A, Lueders C, Busch F, Shayan P, Goel A (2013) Curcumin enhances the effect of chemotherapy against colorectal cancer cells by inhibition of NF-kappaB and Src protein kinase signaling pathways. *PLoS ONE* 8(2):e57218
- Yang SY, Miah A, Sales KM, Fuller B, Seifalian AM, Winslet M (2011) Inhibition of the p38 MAPK pathway sensitises human colon cancer cells to 5-fluorouracil treatment. *Int J Oncol* 38(6):1695–1702
- Cassidy J, Saltz LB, Giantonio BJ, Kabbinavar FF, Hurwitz HI, Rohr UP (2010) Effect of bevacizumab in older patients with metastatic colorectal cancer: pooled analysis of four randomized studies. *J Cancer Res Clin Oncol* 136(5):737–743
- Pang X, Wu Y, Wu Y, Lu B, Chen J, Wang J et al (2011) (-)-Gossypol suppresses the growth of human prostate cancer xenografts via modulating VEGF signaling-mediated angiogenesis. *Mol Cancer Ther* 10(5):795–805
- Li H, Piao L, Xu P, Ye W, Zhong S, Lin SH et al (2011) Liposomes containing (-)-gossypol-enriched cottonseed oil suppress Bcl-2 and Bcl-xL expression in breast cancer cells. *Pharm Res* 28(12):3256–3264
- Gunasekaran GR, Priya DK, Gayathri R, Sakthisekaran D (2011) In vitro and in vivo studies on antitumor effects of gossypol on human stomach adenocarcinoma (AGS) cell line and MNG induced experimental gastric cancer. *Biochem Biophys Res Commun* 411(4):661–666
- Chien CC, Ko CH, Shen SC, Yang LY, Chen YC (2012) The role of COX-2/PGE2 in gossypol-induced apoptosis of colorectal carcinoma cells. *J Cell Physiol* 227(8):3128–3137
- Sadahira K, Sagawa M, Nakazato T, Uchida H, Ikeda Y, Okamoto S et al (2014) Gossypol induces apoptosis in multiple myeloma cells by inhibition of interleukin-6 signaling and Bcl-2/Mcl-1 pathway. *Int J Oncol* 45(6):2278–2286
- Soderquist RS, Danilov AV, Eastman A (2014) Gossypol increases expression of the pro-apoptotic BH3-only protein NOXA through a novel mechanism involving phospholipase A2, cytoplasmic calcium, and endoplasmic reticulum stress. *J Biol Chem* 289(23):16190–16199
- Hsiao WT, Tsai MD, Jow GM, Tien LT, Lee YJ (2012) Involvement of Smac, p53, and caspase pathways in induction of apoptosis by gossypol in human retinoblastoma cells. *Mol Vis* 18:2033–2042
- Jang GH, Lee M (2014) BH3-mimetic gossypol-induced autophagic cell death in mutant BRAF melanoma cells with high expression of p21Cip1. *Life Sci* 102(1):41–48
- Ahn JH, Jang GH, Lee M (2013) Defective autophagy in multidrug resistant cells may lead to growth inhibition by BH3-mimetic gossypol. *J Cell Physiol* 228(7):1496–1505
- Jiang J, Ye W, Lin YC (2009) Gossypol inhibits the growth of MAT-LyLu prostate cancer cells by modulation of TGFbeta/Akt signaling. *Int J Mol Med* 24(1):69–75
- Cengiz E, Karaca B, Kucukzeybek Y, Gorumlu G, Gul MK, Erten C et al (2010) Overcoming drug resistance in hormone- and drug-refractory prostate cancer cell line, PC-3 by docetaxel and gossypol combination. *Mol Biol Rep* 37(3):1269–1277
- Wong FY, Liem N, Xie C, Yan FL, Wong WC, Wang L et al (2012) Combination therapy with gossypol reveals synergism against gemcitabine resistance in cancer cells with high BCL-2 expression. *PLoS ONE* 7(12):e50786
- Sung B, Ravindran J, Prasad S, Pandey MK, Aggarwal BB (2010) Gossypol induces death receptor-5 through activation of

- the ROS-ERK-CHOP pathway and sensitizes colon cancer cells to TRAIL. *J Biol Chem* 285(46):35418–35427
23. Baggstrom MQ, Qi Y, Koczywas M, Argiris A, Johnson EA, Millward MJ et al (2011) A phase II study of AT-101 (gossypol) in chemotherapy-sensitive recurrent extensive-stage small cell lung cancer. *J Thorac Oncol* 6(10):1757–1760
 24. Heist RS, Fain J, Chinnasami B, Khan W, Molina JR, Sequist LV et al (2010) Phase I/II study of AT-101 with topotecan in relapsed and refractory small cell lung cancer. *J Thorac Oncol* 5(10):1637–1643
 25. Formentini A, Henne-Bruns D, Kormmann M (2012) Thymidylate synthase and cyclin D1 protein expression in lymph node negative colorectal cancer: role as prognostic factors? *Hepato-gastroenterology* 59(118):1859–1864
 26. Sprio AE, Di Scipio F, Ceppi P, Salamone P, Di Carlo F, Scagliotti GV et al (2012) Differentiation-inducing factor-1 enhances 5-fluorouracil action on oral cancer cells inhibiting E2F1 and thymidylate synthase mRNAs accumulation. *Cancer Chemother Pharmacol* 69(4):983–989
 27. Vinod BS, Antony J, Nair HH, Puliappadamba VT, Saikia M, Narayanan SS et al (2013) Mechanistic evaluation of the signaling events regulating curcumin-mediated chemosensitization of breast cancer cells to 5-fluorouracil. *Cell Death Dis* 4:e505
 28. Ko JC, Tsai MS, Chiu YF, Weng SH, Kuo YH, Lin YW (2011) Up-regulation of extracellular signal-regulated kinase 1/2-dependent thymidylate synthase and thymidine phosphorylase contributes to cisplatin resistance in human non-small-cell lung cancer cells. *J Pharmacol Exp Ther* 338(1):184–194
 29. Yasuno M, Mori T, Koike M, Takahashi K, Toi M, Takizawa T et al (2005) Importance of thymidine phosphorylase expression in tumor stroma as a prognostic factor in patients with advanced colorectal carcinoma. *Oncol Rep* 13(3):405–412
 30. Ye DJ, Zhang JM (2013) Research development of the relationship between thymidine phosphorylase expression and colorectal carcinoma. *Cancer Biol Med* 10(1):10–15
 31. Jastrzebski K, Hannan KM, Tchoubrieva EB, Hannan RD, Pearson RB (2007) Coordinate regulation of ribosome biogenesis and function by the ribosomal protein S6 kinase, a key mediator of mTOR function. *Growth Factors* 25(4):209–226
 32. Duvel K, Yecies JL, Menon S, Raman P, Lipovsky AI, Souza AL et al (2010) Activation of a metabolic gene regulatory network downstream of mTOR complex 1. *Mol Cell* 39(2):171–183
 33. Magnuson B, Ekim B, Fingar DC (2012) Regulation and function of ribosomal protein S6 kinase (S6K) within mTOR signaling networks. *Biochem J* 441(1):1–21
 34. Hong S, Zhao B, Lombard DB, Fingar DC, Inoki K (2014) Cross-talk between sirtuin and mammalian target of rapamycin complex 1 (mTORC1) signaling in the regulation of S6 kinase 1 (S6K1) phosphorylation. *J Biol Chem* 289(19):13132–13141
 35. Kim SJ, Kim JH, Jung HS, Lee TJ, Lee KM, Chang IH (2014) Phosphorylated p70S6 K in noninvasive low-grade urothelial carcinoma of the bladder: correlation with tumor recurrence. *Asian J Androl* 16(4):611–617
 36. Li J, Xue L, Hao H, Li R, Luo J (2014) Rapamycin combined with celecoxib enhanced antitumor effects of mono treatment on chronic myelogenous leukemia cells through downregulating mTOR pathway. *Tumour Biol* 35(7):6467–6474
 37. Axelrod MJ, Gordon V, Mendez RE, Leimgruber SS, Conaway MR, Sharlow ER et al (2014) p70S6 kinase is a critical node that integrates HER-family and PI3 kinase signaling networks. *Cell Signal* 26(8):1627–1635
 38. Shigematsu H, Yoshida K, Sanada Y, Osada S, Takahashi T, Wada Y et al (2010) Rapamycin enhances chemotherapy-induced cytotoxicity by inhibiting the expressions of TS and ERK in gastric cancer cells. *Int J Cancer* 126(11):2716–2725
 39. Deng J, Lei W, Fu JC, Zhang L, Li JH, Xiong JP (2014) Targeting miR-21 enhances the sensitivity of human colon cancer HT-29 cells to chemoradiotherapy in vitro. *Biochem Biophys Res Commun* 443(3):789–795
 40. Johnson PW (2008) New targets for lymphoma treatment. *Ann Oncol* 19(Suppl 4):iv56–iv59
 41. Kim KW, Kwon HC, Kim SH, Oh SY, Lee S, Lee JH et al (2013) Prognostic significance of thymidylate synthase, thymidine phosphorylase and dihydropyrimidine dehydrogenase expression in biliary tract cancer patients receiving adjuvant 5-fluorouracil-based chemotherapy. *Mol Clin Oncol* 1(6):987–994
 42. Rose MG, Farrell MP, Schmitz JC (2002) Thymidylate synthase: a critical target for cancer chemotherapy. *Clin Colorectal Cancer* 1(4):220–229
 43. Di Cresce C, Figueredo R, Ferguson PJ, Vincent MD, Koropatnick J (2011) Combining small interfering RNAs targeting thymidylate synthase and thymidine kinase 1 or 2 sensitizes human tumor cells to 5-fluorodeoxyuridine and pemetrexed. *J Pharmacol Exp Ther* 338(3):952–963
 44. Bhattacharya B, Akram M, Balasubramanian I, Tam KK, Koh KX, Yee MQ et al (2012) Pharmacologic synergy between dual phosphoinositide-3-kinase and mammalian target of rapamycin inhibition and 5-fluorouracil in PIK3CA mutant gastric cancer cells. *Cancer Biol Ther* 13(1):34–42
 45. Liao F, Yang Z, Lu X, Guo X, Dong W (2013) Sinomenine sensitizes gastric cancer cells to 5-fluorouracil in vitro and in vivo. *Oncol Lett* 6(6):1604–1610
 46. Sprio AE, Di Scipio F, Ceppi P, Salamone P, Di Carlo F, Scagliotti GV et al (2012) Differentiation-inducing factor-1 enhances 5-fluorouracil action on oral cancer cells inhibiting E2F1 and thymidylate synthase mRNAs accumulation. *Cancer Chemother Pharmacol* 69(4):983–989
 47. Di Gennaro E, Bruzzese F, Pepe S, Leone A, Delrio P, Subbarayan PR et al (2009) Modulation of thymidylate synthase and p53 expression by HDAC inhibitor vorinostat resulted in synergistic antitumor effect in combination with 5FU or raltitrexed. *Cancer Biol Ther* 8(9):782–791
 48. Jin HS, Lee DH, Kim DH, Chung JH, Lee SJ, Lee TH (2009) cIAP1, cIAP2, and XIAP act cooperatively via nonredundant pathways to regulate genotoxic stress-induced nuclear factor-kappaB activation. *Cancer Res* 69(5):1782–1791
 49. Murray PE, McNally VA, Lockyer SD, Williams KJ, Stratford IJ, Jaffar M et al (2002) Synthesis and enzymatic evaluation of pyridinium-substituted uracil derivatives as novel inhibitors of thymidine phosphorylase. *Bioorg Med Chem* 10(3):525–530
 50. Evrard A, Cuq P, Robert B, Vian L, Pelegrin A, Cano JP (1999) Enhancement of 5-fluorouracil cytotoxicity by human thymidine-phosphorylase expression in cancer cells: in vitro and in vivo study. *Int J Cancer* 80(3):465–470
 51. Evrard A, Cuq P, Ciccolini J, Vian L, Cano JP (1999) Increased cytotoxicity and bystander effect of 5-fluorouracil and 5-deoxy-5-fluorouridine in human colorectal cancer cells transfected with thymidine phosphorylase. *Br J Cancer* 80(11):1726–1733
 52. Seeliger H, Guba M, Koehl GE, Doenecke A, Steinbauer M, Bruns CJ et al (2004) Blockage of 2-deoxy-D-ribose-induced angiogenesis with rapamycin counteracts a thymidine phosphorylase-based escape mechanism available for colon cancer under 5-fluorouracil therapy. *Clin Cancer Res* 10(5):1843–1852
 53. Nagaraju GP, Alese OB, Landry J, Diaz R, El-Rayes BF (2014) HSP90 inhibition downregulates thymidylate synthase and sensitizes colorectal cancer cell lines to the effect of 5FU-based chemotherapy. *Oncotarget* 5(20):9980–9991
 54. Ohtani K (1999) Implication of transcription factor E2F in regulation of DNA replication. *Front Biosci* 4:D793–D804
 55. Advani SH (2010) Targeting mTOR pathway: a new concept in cancer therapy. *Indian J Med Paediatr Oncology* 31(4):132–136

56. Shuai XM, Han GX, Wang GB, Chen JH (2006) Cyclin D1 anti-sense oligodeoxynucleotides inhibits growth and enhances chemosensitivity in gastric carcinoma cells. *World J Gastroenterol* 12(11):1766–1769
57. Kornmann M, Danenberg KD, Arber N, Beger HG, Danenberg PV, Korc M (1999) Inhibition of cyclin D1 expression in human pancreatic cancer cells is associated with increased chemosensitivity and decreased expression of multiple chemoresistance genes. *Cancer Res* 59(14):3505–3511
58. Wullschlegel S, Loewith R, Hall MN (2006) TOR signaling in growth and metabolism. *Cell* 124(3):471–484
59. Bhattacharya B, Akram M, Balasubramanian I, Tam KK, Koh KX, Yee MQ et al (2012) Pharmacologic synergy between dual phosphoinositide-3-kinase and mammalian target of rapamycin inhibition and 5-fluorouracil in PIK3CA mutant gastric cancer cells. *Cancer Biol Ther* 13(1):34–42
60. Tomioka H, Mukohara T, Kataoka Y, Ekyalongo RC, Funakoshi Y, Imai Y et al (2012) Inhibition of the mTOR/S6 K signal is necessary to enhance fluorouracil-induced apoptosis in gastric cancer cells with HER2 amplification. *Int J Oncol* 41(2):551–558
61. Atreya CE, Ducker GS, Feldman ME, Bergsland EK, Warren RS, Shokat KM (2012) Combination of ATP-competitive mammalian target of rapamycin inhibitors with standard chemotherapy for colorectal cancer. *Invest New Drugs* 30(6):2219–2225
62. O'Reilly T, McSheehy PM, Wartmann M, Lassota P, Brandt R, Lane HA (2011) Evaluation of the mTOR inhibitor, everolimus, in combination with cytotoxic antitumor agents using human tumor models in vitro and in vivo. *Anticancer Drugs* 22(1):58–78
63. Lee KH, Hur HS, Im SA, Lee J, Kim HP, Yoon YK et al (2010) RAD001 shows activity against gastric cancer cells and overcomes 5-FU resistance by downregulating thymidylate synthase. *Cancer Lett* 299(1):22–28
64. Hosono Y, Osada S, Nawa M, Takahashi T, Yamaguchi K, Kawaguchi Y et al (2010) Combination therapy of 5-fluorouracil with rapamycin for hormone receptor-negative human breast cancer. *Anticancer Res* 30(7):2625–2630
65. Dal Col J, Dolcetti R (2008) GSK-3beta inhibition: at the crossroad between Akt and mTOR constitutive activation to enhance cyclin D1 protein stability in mantle cell lymphoma. *Cell Cycle* 7(18):2813–2816
66. Grzybowska-Izydorczyk O, Smolewski P (2012) mTOR kinase inhibitors as a treatment strategy in hematological malignancies. *Future Med Chem* 4(4):487–504
67. Cheng P, Ni Z, Dai X, Wang B, Ding W, Rae Smith A et al (2013) The novel BH-3 mimetic apogossypolone induces Bcl-1- and ROS-mediated autophagy in human hepatocellular carcinoma cells. *Cell Death Dis* 4:e489