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Furowanin A-induced autophagy alleviates apoptosis and promotes cell cycle arrest via inactivation STAT3/Mcl-1 axis in colorectal cancer

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ABSTRACT

Aim

Furowanin A (Fur A) is a flavonoid isolated from *Millettia pachycarpa* Benth. Studies show its potent anti-neoplastic effects against leukemia cells. The aim of the present study was to determine the potential therapeutic effect of Fur A against colorectal cancer (CRC), and elucidate the underlying mechanism.

Material and methods: Cell Counting Kit-8 (CCK-8) assay was used to determine cell, and TUNEL and Annexin-V/PI staining was used to detect apoptosis and the cell cycle distribution. The expression levels of specific proteins in the CRC cells were analyzed by Western blotting. A xenograft model was also established to evaluate the therapeutic effect of Fur A in vivo.

Key findings: Fur A suppressed proliferation, blocked cell cycle progression, induced apoptosis and promoted autophagy in CRC cells. Interestingly, Fur A-induced autophagy functioned not only as a survival mechanism against apoptosis but also intensified the cell cycle arrest in CRC cells. In addition, Fur A mediated its effects via the inactivation of the STAT3/Mcl-1 axis.

Significance: Fur A is a promising drug candidate for the treatment and prevention of CRC.

1. Introduction

Colorectal cancer (CRC) is the second most frequently diagnosed malignancy in women and the third most in men [1], and is responsible for 600,000 deaths worldwide annually [2,3]. Surgical resection is a viable option for patients who are diagnosed at the early stage and present only the localized disease [4]. However, almost a quarter of the CRC patients are diagnosed at the advanced stage when the cancer has already metastasized, at which stage chemotherapy is the main therapeutic regimen. Despite the introduction of novel chemotherapeutics in recent years, the prognosis of patients diagnosed at the advanced stage remains dismal [5]. Therefore, it is necessary to develop novel therapeutic agents with high efficacy and less adverse effects.

Autophagy is a multi-step process that is initiated with the formation of autophagosomes that sequester cellular components, followed by lysosomal fusion and degradation of the cargo in the resulting autophagolysosome [6]. Various factors, including mitochondrial loss, oxidative stress, nutrient deficiency and toxic chemicals, can trigger autophagy [7]. Autophagy plays a context-dependent and tumor-specific role in the development and progression of cancer. It functions as a tumor suppressor [8] by inducing cell death, either in conjunction with apoptosis or as a standby mechanism in case the former is defective [9,10]. However, some cancer cells also use autophagy as a survival mechanism to maintain homeostasis during starvation or other stress, leading to tumor progression [11].

A number of naturally occurring compounds are known exert anti-tumor effects by modulating autophagy [12,13]. *Millettia pachycarpa* Benth (Houguojixueteng in Chinese), a medicinal plant widely distributed in the Southwest part of China, has been used widely as a folk medicine to clear parasitic intestinal worms [14]. The anti-inflammatory effects of its extract have also been observed in vitro [14]. A number of flavonoid and isflavonoid compounds have been identified as the active ingredients of *M. pachycarpa* Benth. Furowanin A (Fur A), a isoflavonoid compound extracted from its leaves, induces apoptosis in leukemic HL-60 cells [15]. However, the potential anti-cancer effect of Fur A against solid tumors is unknown. In the current study, we investigated the effect Fur A on cell proliferation, cell cycle distribution, apoptosis and autophagy in vitro and in vivo, as well as the underlying mechanisms. We found that Fur A promoted autophagy in CRC cells, which in turn reversed its pro-apoptotic effects and further augmented the cell cycle blockade. Furthermore, our study provides experimental evidence that Fur A exerts its effects via the STAT3/Mcl-1 axis.

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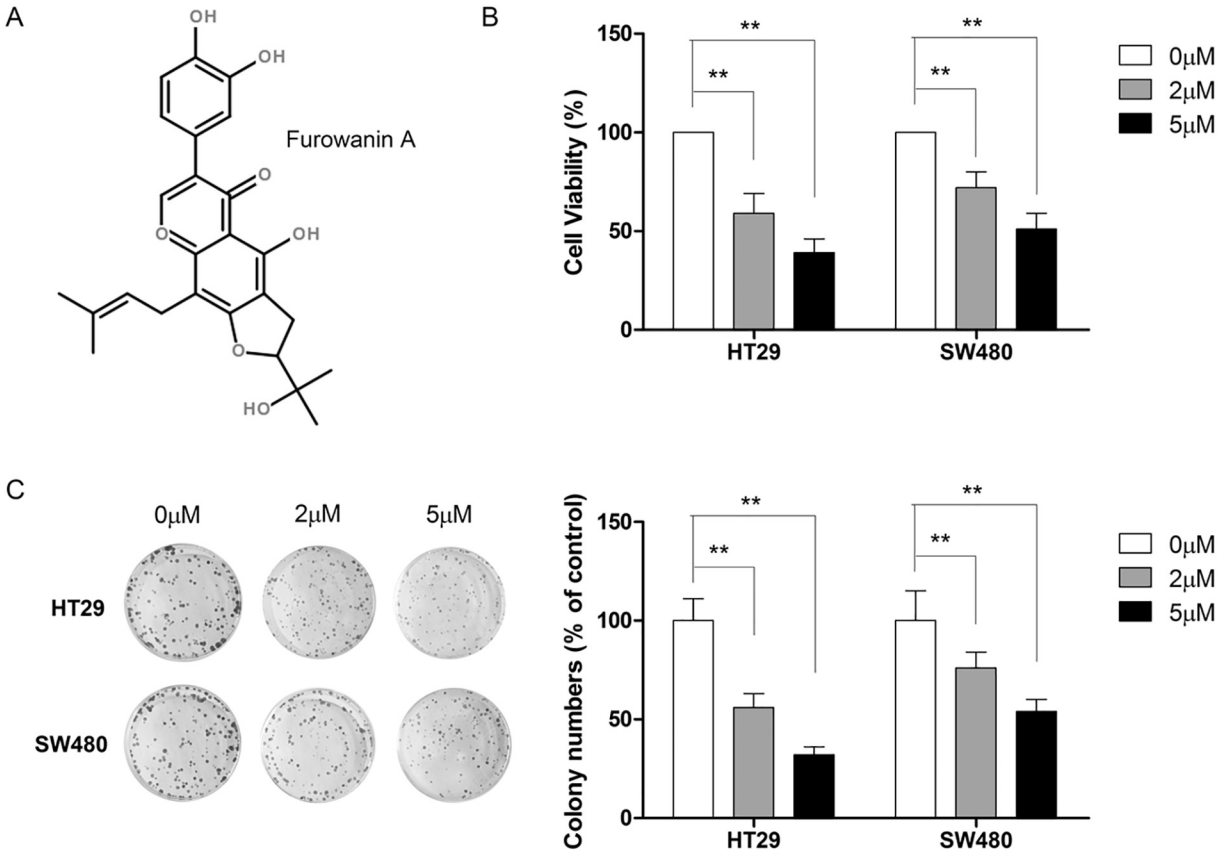

Fig. 1. Fur A inhibits growth of HT29 and SW480 cells. A. Chemical structure of Fur A. B. CRC cells treated with Fur A for 48 h had significantly reduced viability. C. Fur A inhibited anchorage-independent growth of CRC cells. **P < 0.01.

2. Materials and methods

2.1. Cell lines and culture conditions

The human colon cancer cell lines (HT29 and SW480) and human normal colon epithelial cell line (NCM460) were provided by the Centre for Cell Resources of Shanghai Institute for Life Sciences, Chinese Academy of Sciences (Shanghai, China). Both lines were cultured in RPMI-1640 medium containing 10% fetal bovine serum, 25 mg/ml amphotericin B, 100 U/ml penicillin and 100 mg/ml streptomycin, at 37 °C in a humidified incubator with 5% CO2.

2.2. Cell counting Kit-8 (CCK-8) assay

To identify the optimal cytotoxic concentration of Fur A, CRC cells were treated with 0, 2, 5 and 10 μM Fur A (dissolved in DMSO, diluted in using medium) for 24 h and 48 h. Briefly, the cells were seeded into 96-well plates at the density of 5×10^3 cells/well and after culturing for the stipulated durations, 10 μl CCK-8 solution was added per well, and the cells were incubated for another 2 h. The absorbance was measured at 450 nm using a microplate reader (BioTek, Winooski, VT, USA).

2.3. Colony formation assay

CRC cells treated with different concentrations of Fur A (0, 2 and 5 μM) were seeded into six-well plates at the density of 600 cells/well and cultured for 2 weeks without changing the medium. The ensuing colonies (aggregates of > 50 cells) were fixed with paraformaldehyde for 15 min and stained with 1% crystal violet for 10 min. The number of colonies was counted manually under a light microscope with the help of Image-Pro Plus 6.0 software (Media Cybernetics, MD).

2.4. Apoptosis assays

Following exposure to Fur A (0, 2 and 5 μM) for 48 h, CRC cells were stained with the TUNEL dye (Beyotime, Wuxi, China) according to the manufacturer's instructions. At least 300 cells in five random fields (100× magnification) per sample were counted to calculate the ratio of TUNEL positive apoptotic cells. In addition, transfected CRC cells were harvested and double stained using Annexin V-FITC/PI for 15 min. The stained cells were observed by flow cytometry, and the percentage of apoptotic cells was calculated.

2.5. Cell cycle analysis

CRC cells were cultured in RPMI 1640 medium containing 10% FBS for 48 h, and sub-cultured at the density of 1×10^5/well in a 6-well culture plate. 3-MA was purchased from sigma (M9281-100MG). The concentration of 3-MA used was 5 mM (6 h). To evaluate the effect of Fur A on cell cycle distribution, CRC cells were exposed to different concentrations of Fur A (2 and 5 μM) for 48 h, washed, and fixed with ice-cold 70% ethanol at 4 °C for 2 h. FACScan DNA analysis was performed following standard protocol.

2.6. Western blotting

RIPA sample buffer (Beyotime, China) supplemented with protease inhibitors (Complete, EDTA-free; Roche, USA) and PMSF (Beyotime, China) was used to extract total proteins from cells and tissues. Equal amounts (30 μg) of protein per sample were separated by SDS-PAGE, and transferred onto a PVDF membrane. The latter were blocked with

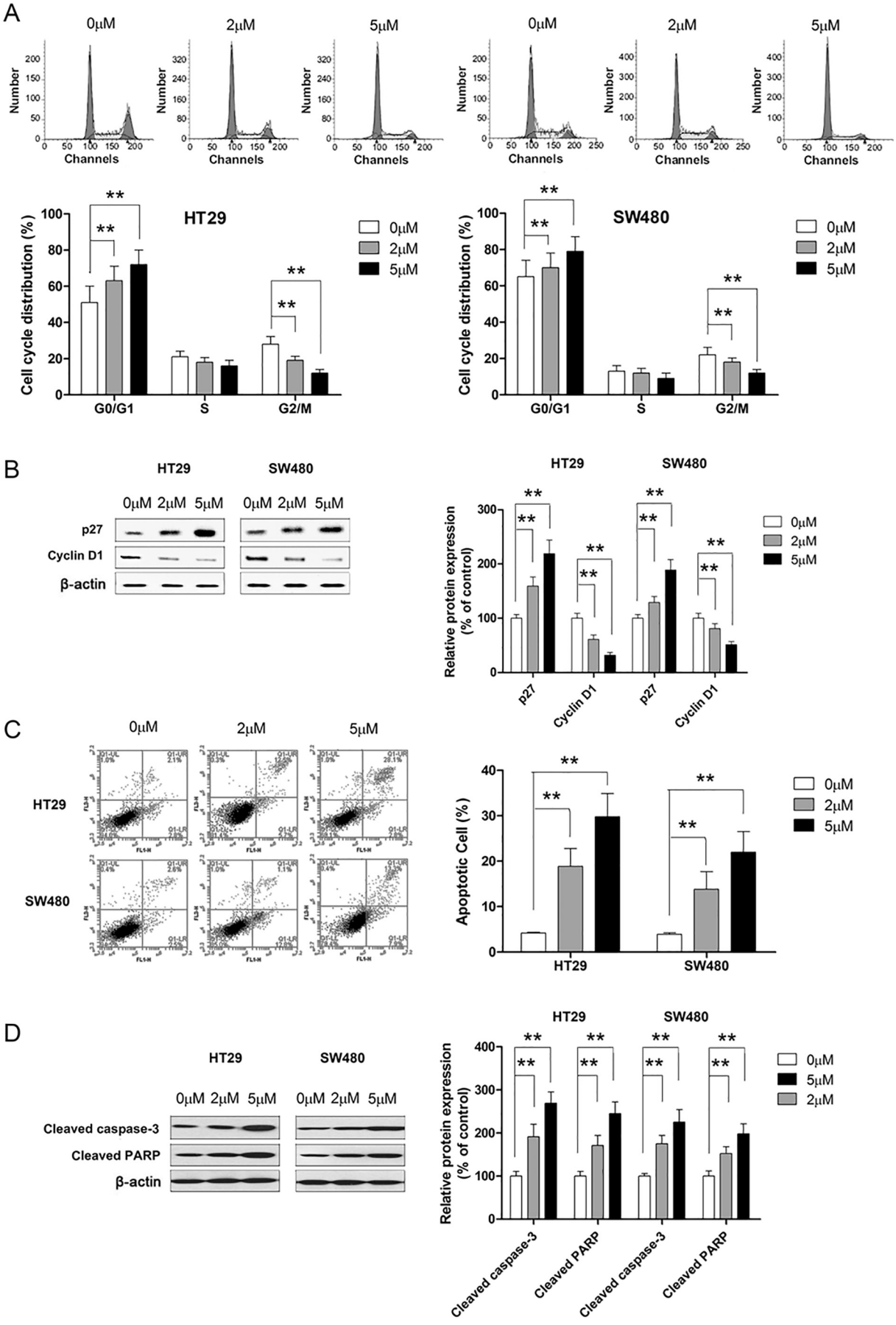

Fig. 2. Fur A blocks cell cycle progression and promotes apoptosis in HT29 and SW480 cells. Fur A increased the proportion of cells at the G0/G1 phase and decreased that at the G2/M phase (A), upregulated p27 and downregulated Cyclin D1 (B), promoted apoptosis (C), and activated caspase-3 and PARP (D).

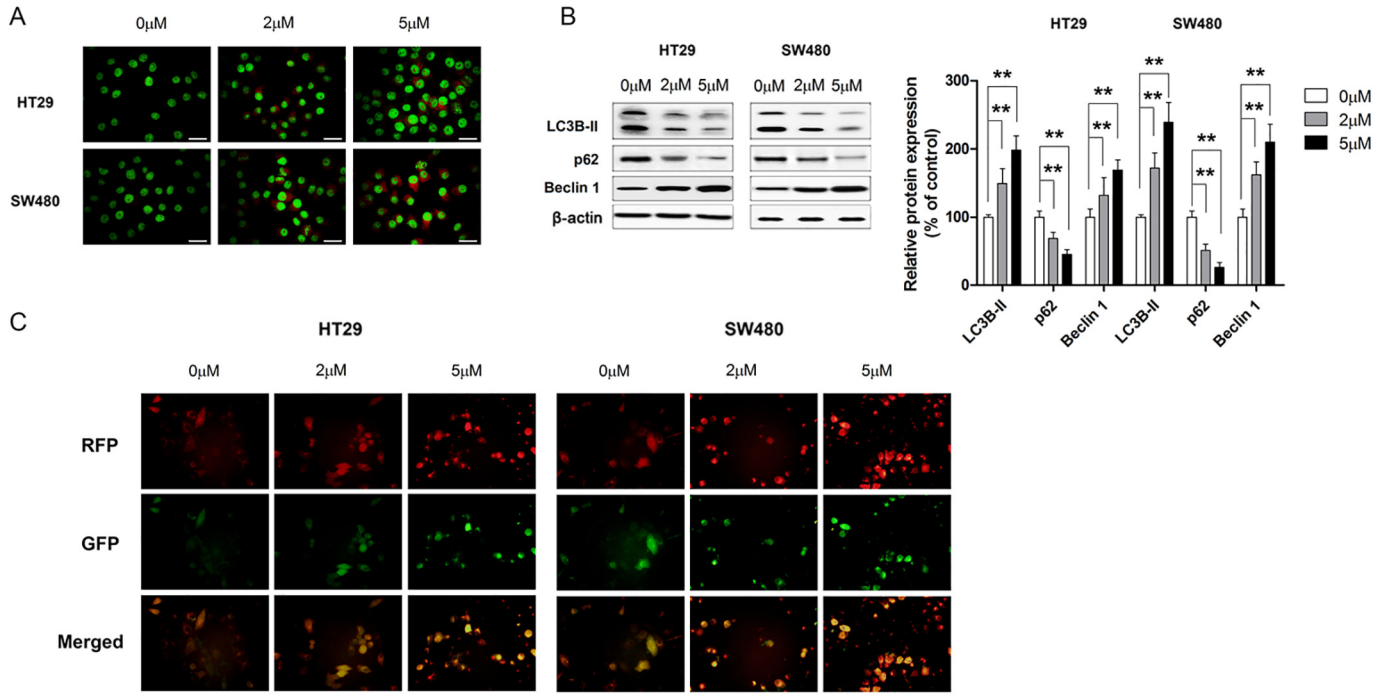

Fig. 3. Fur A promotes autophagy in HT29 and SW480 cells. Fur A induced formation of acidic vesicle organelles (AVOs) (A), upregulated the autophagy-related proteins LC3B-II and Beclin 1 and downregulated p62 (B), and promoted formation of autophagosomes (yellow puncta) and autolysosomes (red puncta) (C). **P < 0.01. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

5% non-fat milk, and incubated overnight with specific primary antibodies against p27 (#3688, 1:1000), Cyclin D1 (#2922, 1:1000), cleaved-caspase-3 (#9661. 1:1000), cleaved PARP (#5625, 1:1000), LC3B (#2775, 1:1000), Beclin-1 (#3738, 1:1000; all from Cell Signaling Technology, Danvers, MA), p62 (#56416, 3 μg/ml), p-STAT3-Y705 (#76315. 1:2000), STAT3 (#119352, 1:3000), Mcl-1 (#32087, 1:3000; all from Abcam, Cambridge, MA, USA), and the internal control β-actin (Beyotime Biotechnology, Shanghai, China) at 4 °C. The immunopositive bands were probed using HRP-labeled goat anti-rabbit IgG (#A0208, 1:1000) or anti-mouse IgG (#A0216, 1:1000) for 1 h at room temperature. The positive bands were visualized by enhanced chemiluminescent (ECL) detection reagent (Thermo Fisher, USA), and the images were analyzed and quantified using Gel Doc 2000 (BioRad, USA).

2.7. Acridine orange staining

The development of autophagic acidic vesicle organelles (AVO) was detected by acridine orange staining. Briefly, the cells were treated with different concentrations of Fur A (0, 2 and 5 μM) for 24 h and washed thrice with PBS. After staining with 0.01% acridine orange (Solarbio, China) for 5 min, the cells were observed under a fluorescence microscope through the red filter (BX53, OLYMPUS, Japan).

2.8. Transduction with the mRFP-GFP-LC3 adenovirus

CRC cells were transduced with mRFP-GFP-LC3 adenovirus (Hanbio, China), and treated with different concentrations of Fur A (0, 2 and 5 μM). Autolysosomes were detected 48 h later using a confocal microscope under 400× magnification. The yellow and red puncta were respectively the autophagosomes and autolysosomes.

2.9. Transfection of CRC cells

CRC cells were transfected with STAT3 expression plasmid (and the empty vector), or with siRNAs targeting STAT3/Beclin 1 (and the non-targeting scrambled siRNAs) (Santa Cruz, CA) using Lipofectamine 3000 and Lipofectamine 2000 respectively (Invitrogen). The medium was replaced 48 h after transfection, and the cells were harvested for suitable assays.

2.10. In vivo tumor modelling

All animal experiments were approved by the Medical Ethics Committee of the People's Hospital of Zhengzhou University. Eight-week-old male athymic BALB/c nu/nu mice were subcutaneously injected with HT29 cells (1×10^7 cells/mL) on their left flanks. Fifteen days after injection, the mice were randomly divided into the following three groups (n = 6) that received intraperitoneal (IP) injections of: (A) vehicle (0.9% sodium chloride and 1% DMSO), (B) low dose Fur A (20 mg/kg/day), and (C) high dose Fur A (40 mg/kg/day). The body weight of the mice and tumor volumes were measured every three days. After treatment for 30 days, the tumors were harvested and subjected to protein extraction for Western blotting, or cut into 4 μm cryostat sections for H&E and TUNEL staining as previously described [16]. The acute toxicity of Fur A (20 and 40 mg/kg/day) against heart, liver, lung and renal tissues was evaluated using H&E staining.

2.11. Statistical analysis

All statistical analyses were conducted using SPSS software 16.0 (SPSS Inc., Chicago, IL). One-way ANOVA followed by Dunnett's *t*-test was used to compare cell lines, and p values < 0.05 were considered statistically significant.

3. Results

3.1. Fur A suppresses CRC cell proliferation and anchorage-independent growth

The chemical structure of Fur A is shown in Fig. 1A. As shown in Supplementary Fig. 1, treatment of the CRC cell lines with 0, 1, 2, 5 and

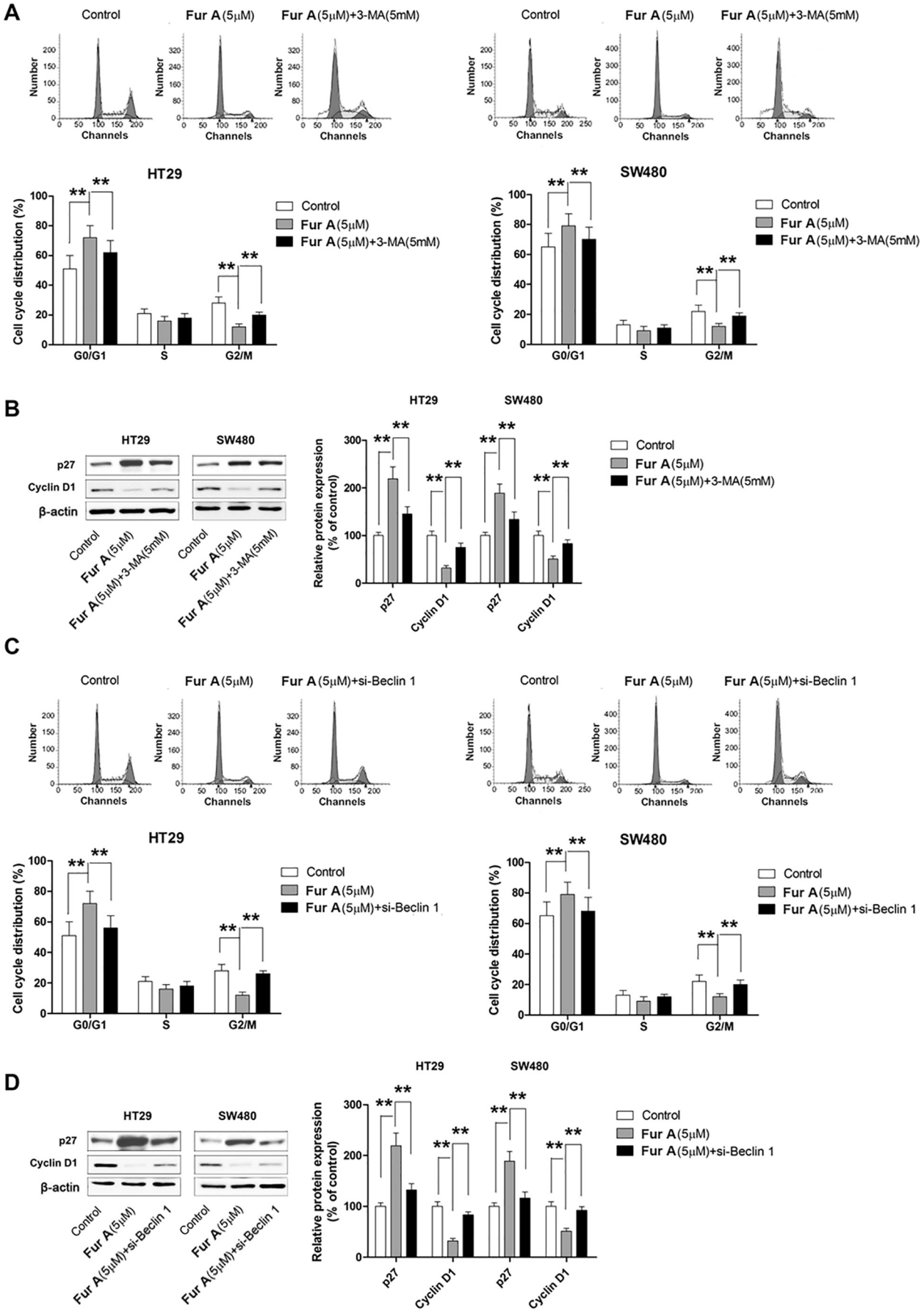

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Fig. 4. Autophagy inhibition by 3-MA or si-Beclin 1 abrogates Fur A-induced cell cycle blockade. Pre-treatment with 3-MA (3 mM, 6 h before Fur A treatment) reversed Fur A-induced skewed cell cycle (A) and attenuated Fur A-induced upregulation of p27 and downregulation of Cyclin D1 (B). Beclin 1 knockdown reversed Fur A-induced cell cycle blockade (C) and attenuated Fur A-induced upregulation of p27 and downregulation of Cyclin D1 (D). **P < 0.01.

10 μM Fur A for 24 h did not significantly affect their viability, except in SW480 cells treated with 10 μM of the drug. After the 48 h treatment, Fur A significantly suppressed HT-29 and SW480 cell growth at all doses (**$P < 0.01$), while 10 μM Fur A was also toxic on the normal colon epithelial NCM460 cells. Therefore, we selected 2 and 5 μM Fur A for the subsequent experiments. Following a 48 h incubation with 2 μM Fur A, the viability of the HT29 and SW480 cells decreased to 59 ± 8.6% and 72 ± 10.3% respectively (Fig. 1B); with 5 μM Fur A, the percentage of viable cells dropped further to 39 ± 7.2% and 51 ± 6.2% (Fig. 1B). The effect of Fur A on the anchorage-independent growth of CRC cells was evaluated using colony formation assay. After culturing the cells for 2-weeks in the presence of 2 μM Fur A, the number of HT29 and SW480 colonies respectively decreased to 56 ± 6.8% and 74 ± 8.3% relative to the untreated controls (Fig. 1C).

3.2. Fur A promotes apoptosis and cell cycle arrest in CRC cells

As shown in Fig. 2A, Fur A increased the proportion of cells in the G0/G1 phase, and decreased that in the G2/M phase in a dose-dependent manner, indicating cell cycle arrest at the G0/G1 phase. Fur A treatment at the concentrations of 2 μM and 5 μM resulted in the accumulation of respectively 70% and 80% of the SW480 cells in the G1/G0 phase (Fig. 2A). Consistent with this, Fur A markedly elevated the expression levels of p27 and repressed that of cyclin D1 in both cell lines (Fig. 2B). Furthermore, 2 and 5 μM Fur A increased the apoptotic HT29 cell population by 18.9 ± 2.9% and 29.8 ± 4.3% respectively (Fig. 2C), while the SW480 cells were more resistant to the pro-apoptotic effects of Fur A (Fig. 2C). To consolidate our results, we also tracked the activation of caspase-3 and PARP proteins, and found that Fur A activated both in a dose-dependent manner (Fig. 2D). Taken together, Fur A exerted an anti-proliferative effect on the CRC cells by triggering cell cycle arrest and apoptosis.

3.3. Fur a promotes autophagy in CRC cells

To analyze the effect of Fur A on autophagy, we stained the CRC cells with acridine orange to visualize the acidic vesicular organelles (AVOs), a morphological feature of autophagy. Fur A treatment resulted in a marked increase in red fluorescence signals, indicating numerous AVOs (Fig. 3A). In addition, Fur A significantly upregulated the pro-autophagic LC3BII and Beclin 1 proteins, and down-regulated p62 (Fig. 3B). Fur A also significantly increased the number of autophagosomes and autolysosomes in the CRC cells in a dose-dependent manner, indicating a higher autophagy flux (Fig. 3C).

3.4. Inhibition of autophagy blocks Fur A–induced cell cycle arrest and promotes apoptosis

Autophagy is known to regulate cell cycle progression and survival [17]. To determine the role of Fur A-induced autophagy in CRC cells, we treated the cells with 3-MA, a specific autophagy inhibitor. Pre-treatment with 3-MA significantly blocked autophagy (Supplementary Fig. 2A) and reversed the Fur A-induced cell cycle arrest at G0/G1 phase (Fig. 4A), p27 induction, and cyclin D1 suppression (Fig. 4B). Furthermore, 3-MA treatment alone affected neither the cell cycle distribution nor apoptosis (Supplementary Fig. 2B–C). Autophagy was also inhibited at the genetic level via siRNA-mediated Beclin 1 knockdown (Supplementary Fig. 3A). As shown in Fig. 4C, Beclin 1 inhibition abrogated the Fur A-induced cell cycle arrest, and reversed Fur A-induced alterations in p27 and Cyclin D1 expression (Fig. 4D). In addition, no significant changes were observed in cell cycle progression and apoptosis following Beclin 1 knockdown alone (Supplementary Fig. 3B–C). Interestingly, pharmacological inhibition of autophagy significantly increased apoptosis in HT29 cells from 29.80% to 36.2% ($P < 0.01$) and sensitized the SW480 cells to Fur A-induced apoptosis (Fig. 5A), in addition to further enhancing caspase-3 and PARP activation (Fig. 5B). Consistent with this, Beclin 1 knockdown also augmented the pro-apoptotic effects of Fur A in both cell lines (Fig. 5C), and upregulated cleaved caspase-3 and PARP levels (Fig. 5D). Taken together, Fur A-induced autophagy further strengthened the cell cycle arrest in CRC cells, while protecting them against apoptosis.

3.5. Fur A promotes cell cycle arrest, apoptosis and autophagy by modulating the STAT3/Mcl-1 axis

Since STAT3/Mcl-1 is known to regulate various biological activities in cancer cells, for e.g. cell cycle progression, survival and autophagy [18,19], we determined whether Fur A also affected STAT3 expression levels and/or activity. As shown in Fig. 6A, Fur A markedly suppressed the level of phosphorylated STAT3, indicating the inhibition of the STAT3 signaling pathway. Furthermore, Fur A also down-regulated Mcl-1, a key target gene of STAT3 [20], in a dose-dependent manner in both HT29 and SW480 cells (Fig. 6A). To further validate the role of STAT3/Mcl-1 in the anti-neoplastic action of Fur A, HT29 and SW480 cells were transfected with a STAT3-overexpressing plasmid (Fig. 6B). The ectopic expression of STAT3 in CRC cells not only blocked Fur A-induced repression of Mcl-1 (Fig. 6B), but also abolished the cell cycle arrest (Fig. 6C) and changes in p27 and cyclin D1 expression (Fig. 6D) induced by Fur A. In addition, ectopic STAT3 expression also blocked Fur A-induced elevation of LC3BII and Beclin 1 and repression of p62 in both HT29 and SW480 cells (Fig. 6D), indicating that Fur A triggers autophagy by inhibiting the STAT3 signaling pathway. Finally, ectopic STAT3 expression rescued CRC cells from Fur A-induced apoptosis (Fig. 6E), and attenuated caspase-3 and PARP activation (Fig. 6F). To further validate the role of STAT3 signaling in Fur A action, HT29 and SW480 cells were transfected with STAT3 siRNA (Fig. 7A). STAT3 knockdown in Fur A treated cells markedly repressed Mcl-1 levels (Fig. 7A), intensified the cell cycle arrest (Fig. 7B), augmented p27 upregulation and cyclin D1 down-regulation (Fig. 7C), enhanced autophagy in terms of high LC3BII and Beclin 1 and low p62 levels (Fig. 7C), and sensitized the cells to apoptosis (Fig. 7D and E). Taken together, STAT3/Mcl-1 signaling mediates Fur A-induced cell cycle arrest, apoptosis and autophagy.

3.6. Fur A suppresses HT29 xenograft tumor growth

To examine the anti-tumor effect of Fur A in vivo, an HT29 xenograft tumor model was established and treated with 20 or 40 mg/kg Fur A everyday, and the size of the tumors were examined every 3 days. Both doses of Fur A suppressed tumor growth (Fig. 8A), without causing any significant weight loss in the mice, indicating that Fur A was generally well tolerated by the mice (Fig. 8B). In addition, Fur A treatment increased the percentage of TUNEL positive apoptotic cells in the tumor tissues in a dose-dependent manner (Fig. 8C). The Fur A-induced tumor growth inhibition was associated with an increase in the levels of cleaved caspase-3, LC3BII, Beclin 1 and p27 (Fig. 8D), and a dose-dependent decrease in that of Ki67, pSTAT3, Mcl-1, p62 and cyclin D1 (Fig. 8D). To evaluate any potential side effects of Fur A, the major organs – heart, liver, spleen, lung, and kidney – were examined histologically by H&E staining, and no significant pathological changes were observed with either dose (Supplementary Fig. 4). Taken together, Fur A inhibited tumor growth in vivo by inducing apoptosis and autophagy

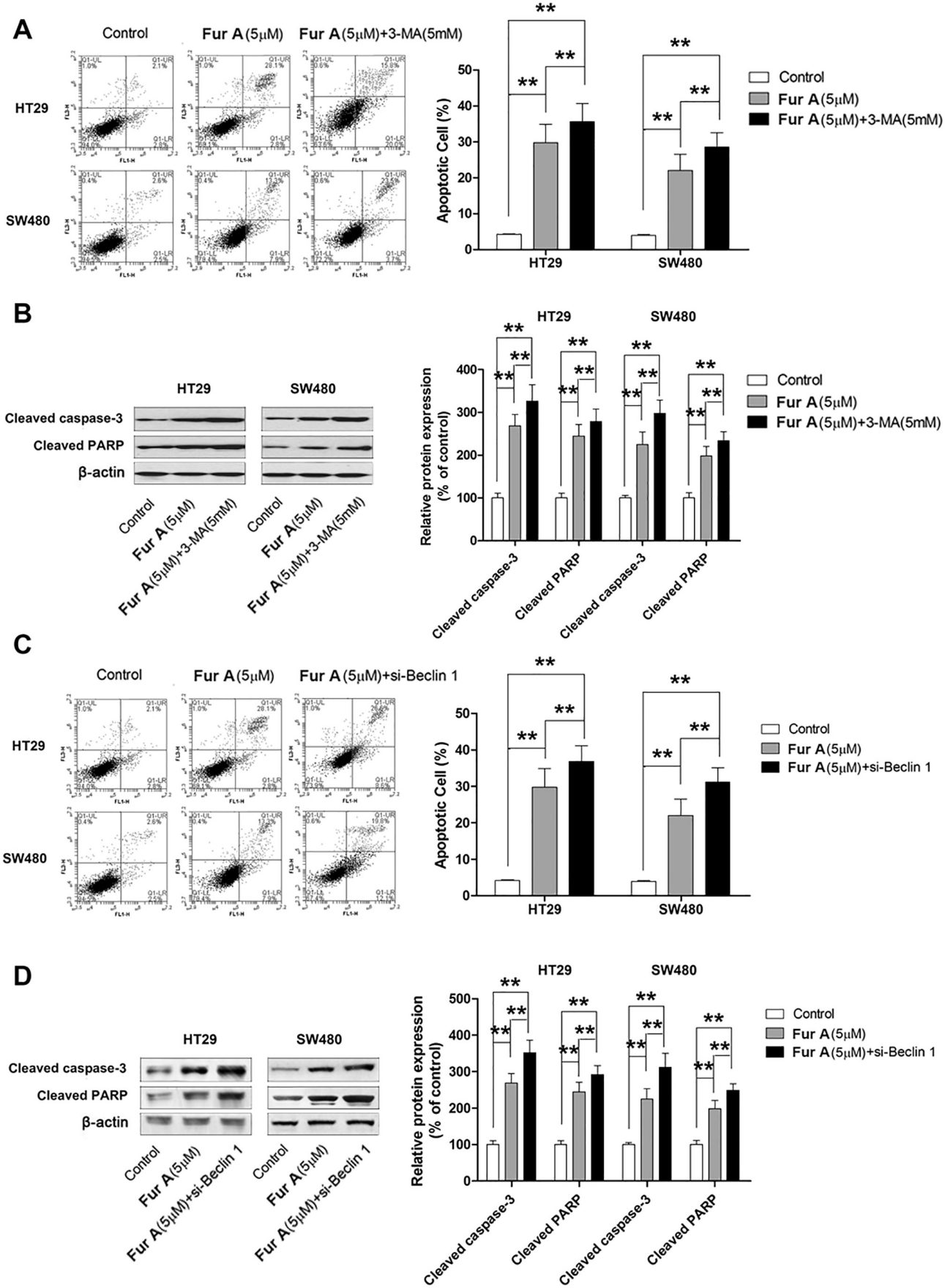

Fig. 5. Autophagy inhibition by 3-MA or si-Beclin 1 augments Fur A-induced apoptosis. Pre-treatment with 3-MA enhanced Fur A-induced apoptosis (A) and augmented Fur A-induced activation of caspase-3 and PARP (B). Beclin 1 knockdown enhanced Fur A-induced apoptosis (C) and augmented Fur A-induced activation of caspase-3 and PARP (D). **P < 0.01.

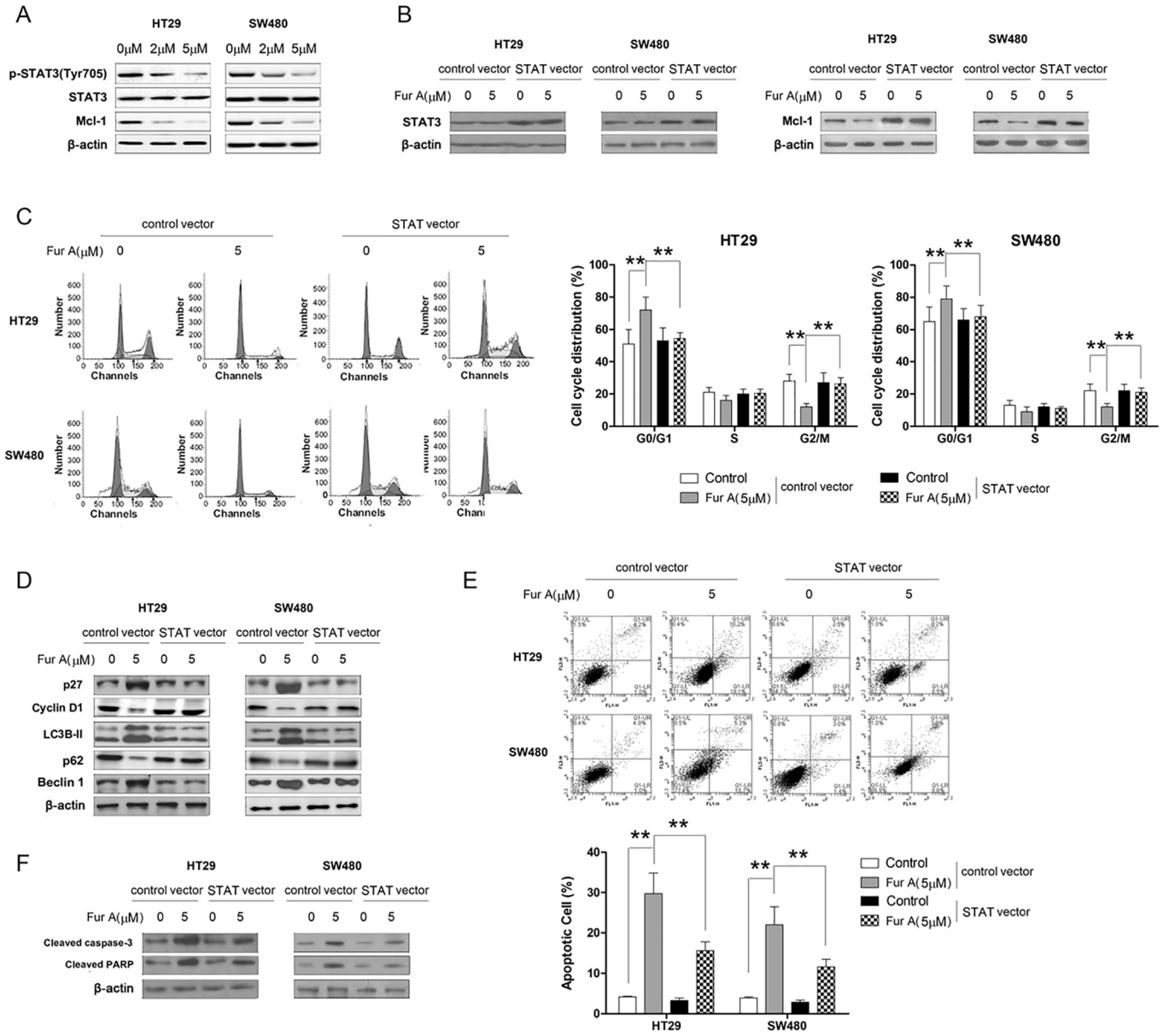

Fig. 6. Ectopic expression of STAT3 abrogates Fur A-induced cell cycle arrest, apoptosis and autophagy. A. Fur A inhibited STAT3 activation and repressed Mcl-1 expression B. Ectopic expression of STAT3 upregulates Mcl-1 expression. Effect of STAT3 overexpression on Fur A-induced cell cycle arrest (C), p27, Cyclin D1, p62, LC3BII and Beclin 1 expression (D), Fur A-induced apoptosis (E), and Fur A-induced activation of caspase-3 and PARP (F). **P < 0.01.

in the tumor cells, without affecting the normal tissues.

4. Discussion

Fur A is one of the active ingredients in *M. pachycarpa* Benth, a herb used in traditional Chinese medicine preparations [14]. Although the anti-neoplastic effects of Fur A have been observed in the leukemic cell line HL-60 [15], little is known about its effects in solid tumors, as well as the exact molecular mechanism(s). We found that Fur A suppressed proliferation of CRC cells by blocking cell cycle progression and promoting apoptosis. This is the first study to report a pro-autophagic function of Fur A in cancer cells, which promoted Fur A-induced cell cycle arrest but protected the cells against apoptosis. Furthermore, we also provided experimental evidence of the mechanistic role of STAT3/Mcl-1 signaling pathway in mediating the effects of Fur A in CRC cells.

Abnormal cell cycle progression is one of the cardinal features of malignant cells [21]. The cell cycle checkpoints are tightly regulated by cyclins, cyclin-dependent kinases (CDKs) and cyclin-dependent kinase inhibitors (CKIs), which control transition through the different cell cycle phases [21]. The p27 and cyclin D1 proteins are crucial factors that control cellular entry into the S phase [22]. Fur A blocked cell cycle progression at the G0/G1 phase, with a concomitant decrease in the proportion of cells at the G2 phase, by upregulating p27 and downregulating cyclin D1. A number of recent studies have addressed the complex link between autophagy and cell survival, although the mechanisms underlying the autophagic control of tumor cell proliferation, apoptosis and cell cycle progression have not been elucidated [23,24]. Therefore, drugs or natural compounds that autophagy can potentially target tumor cells. For instance, Luo et al. have reported that autophagy induction by Gartanin, a natural xanthone of mangosteen, promoted cell cycle arrest at the G1/G0 phase in glioma cells [25], while inhibition of autophagy induced by metformin rescued myeloma cells from G1/G0 phase blockade [26]. In accordance with these studies, we found that autophagy induced by Fur A promoted G0/G1 arrest in the CRC cells.

Apoptosis, or programmed cell death I, is the mechanistic basis of

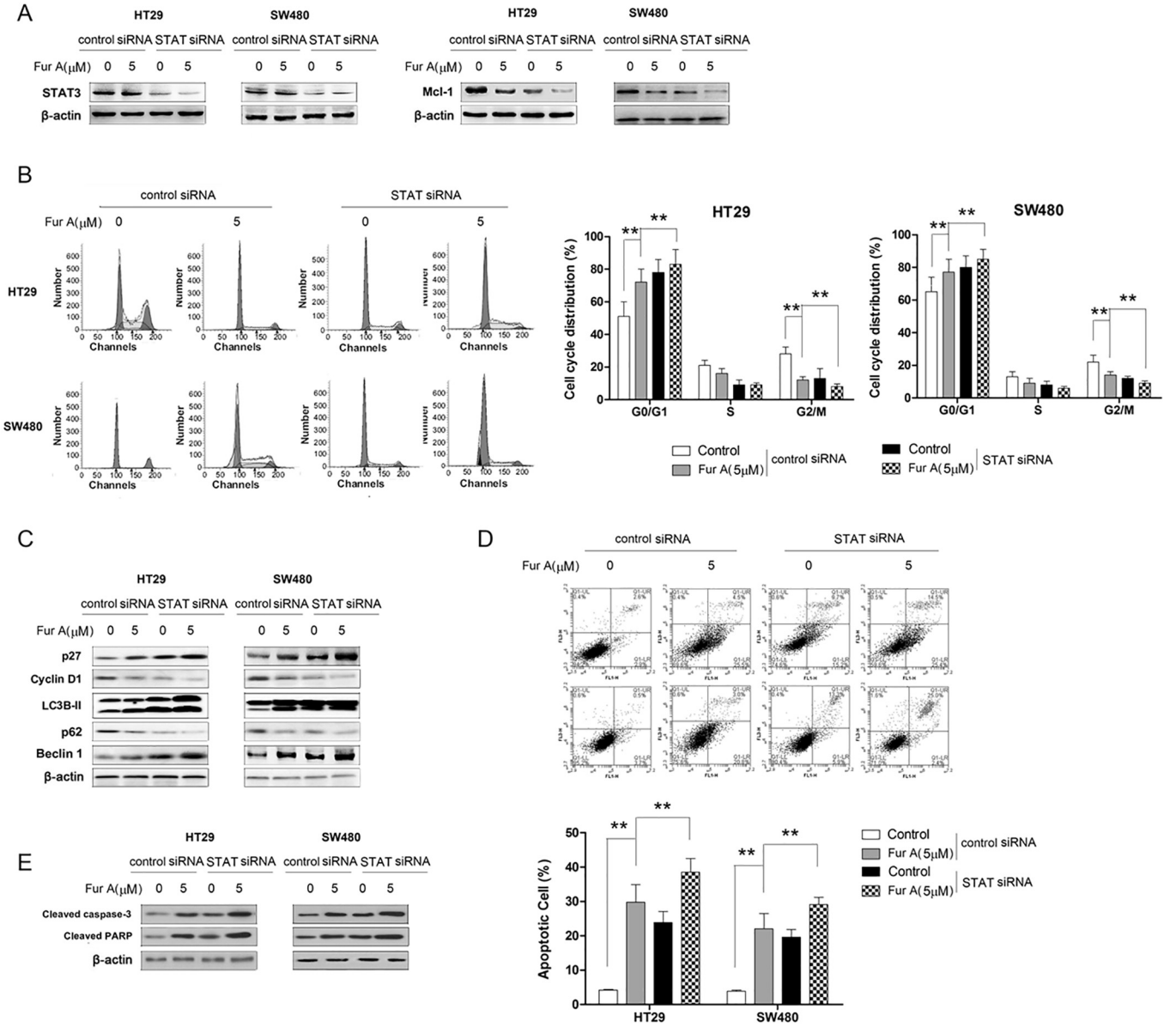

Fig. 7. STAT3 knockdown augments Fur A-induced cell cycle arrest, apoptosis and autophagy. A. STAT3 knockdown downregulated Mcl-1 expression. Effect of STAT3 knockdown on Fur A-induced cell cycle arrest (C), p27, Cyclin D1, p62, LC3BII and Beclin 1 expression (D), Fur A-induced apoptosis (E), and Fur A-induced activation of caspase-3 and PARP (F). **P < 0.01.

most anti-cancer chemotherapeutic agents [27]. Fur A also promoted apoptosis in the CRC cell lines as well as the tumor tissues, indicating that it exerts its anti-neoplastic effects at least partly via apoptosis. Several naturally occurring flavonoids have been shown to induce apoptosis and autophagy in tumor cells simultaneously [28,29]. However, autophagy can either function as a protective mechanism against apoptosis or as its very trigger in cancer cells. For example, autophagy induced by icaritin promoted apoptosis in glioma cells [30], as did that induced by luteolin in hepatocellular carcinoma cells [31]. In contrast, induction of protective autophagy against apoptosis has also been observed in cancer cells treated with different flavonoid compounds such as nobiletin [32], quercetin [33] and myricetin [34]. Pre-treatment with the autophagy inhibitor 3-MA, as well as Beclin 1 knockdown, enhanced Fur A-induced apoptosis in CRC cells. These findings strongly indicate a pro-survival effect of Fur A-induced autophagy against apoptotic stimuli.

Studies show the constitutive activation of STAT3 signaling in numerous cancer types, including CRC [35]. STAT3 promotes carcinogenesis either directly by transcriptionally regulating onco/tumor-suppressor genes, or indirectly via chromatin remodeling [36]. In addition, STAT3 is involved in essential cellular functions related to proliferation, survival, and angiogenesis [37]. STAT3 and its downstream genes like Mcl-1 regulate the expression of genes involved in cancer cell proliferation, survival and autophagy [38]. In accordance with previous findings [19,39], ectopic expression STAT3 in CRC cells up-regulated Mcl-1, and abolished Fur-A induced cell cycle arrest and apoptosis. On the other hand, STAT3 knockdown repressed Mcl-1 and sensitized the CRC cells to cell cycle blockade and apoptosis induced by Fur A. These findings indicate that the anti-neoplastic effects of Fur A are mediated by STAT3/Mcl-1. This is consistent with the finding that Mcl-1 is able to regulate autophagy by forming a complex with Beclin 1 [40]. Furthermore, suppression of STAT3/Mcl-1 signaling is the mechanistic basis of autophagy induced by a few chemotherapeutic agents [41,42]. We found that while ectopic expression of STAT3 markedly attenuated Fur A-induced autophagy, it was enhanced upon STAT3 knockdown. Altogether, these results indicate that STAT3/Mcl-1

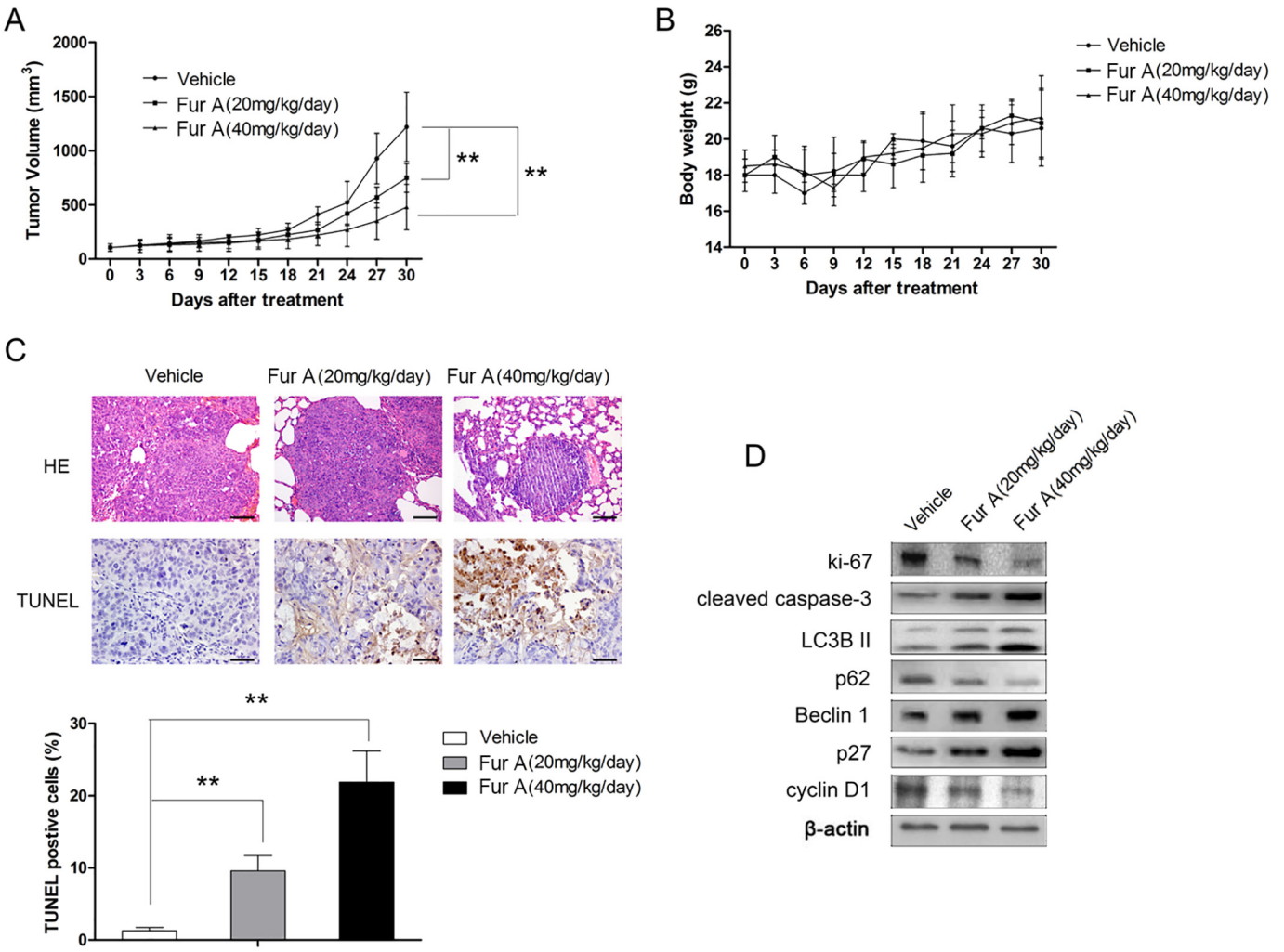

Fig. 8. Fur A suppresses tumor growth in the HT29 xenograft mice model. A. Fur A inhibited tumor growth and reduced the tumor volume. B. Fur A treatment did not result in systemic toxicity and weight loss. C. HE and TUNEL staining of xenograft tumor tissue. D. Western blotting image showing levels of various signaling proteins. **P < 0.01.

inhibition by Fur A is at least partly responsible for its pro-autophagic effects.

In summary, our findings show that Fur A suppresses CRC cell proliferation and anchorage-dependent growth via cell cycle arrest, apoptosis and autophagy, and Fur A-induced autophagy protects against apoptosis while triggering cell cycle arrest. Furthermore, these effects of Fur A are at least partially mediated by inhibition of the STAT3/Mcl-1 pathway. Although the mechanisms involved in Fur A-mediated anti-tumor action need to be elucidated further, our results have provided a preliminary basis to investigate the therapeutic potential of Fur A in cancer.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2018.12.027.

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Conflict of interest

None.

Author contribution

Xue-bin BAO designed the research; Zhao MA and Xue-bin BAO performed experiments; Jun-bao GU analyzed data and wrote the paper.

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