ORIGINAL RESEARCH

RETRACTED ARTICLE: 5-Fluorouracil Combined with Rutaecarpine Synergistically Suppresses the Growth of Colon Cancer Cells by Inhibiting STAT3

Zhen Yu [1],*, Shixin Chan[2],*, Xu Wang[2], Rui Sun[2], Ming Wang [1], Zhenglin Wang[2], Xiaomin Zuo [2], Jiajie Chen[3], Huabing Zhang[4], Wei Chen[1,2,5]

[1]Department of General Medicine, The First Affiliated Hospital of Anhui Medical University, Hefei, 230022, People's Republic of China; [2]Department of General Surgery, The First Affiliated Hospital of Anhui Medical University, Hefei, 230022, People's Republic of China; [3]Department of Dermatology, First Affiliated Hospital of Anhui Medical University, Hefei, 230022, People's Republic of China; [4]Department of Biochemistry & Molecular Biology, School of Basic Medicine, Anhui Medical University, Hefei, 230032, People's Republic of China; [5]Anhui Provincial Institute of Translational Medicine, Hefei, 230022, People's Republic of China

*These authors contributed equally to this work

Correspondence: Huabing Zhang, Department of Biochemistry & Molecular Biology, School of Basic Medicine, Anhui Medical University, Hefei, 230032, People's Republic of China, Email slzhang1977@163.com; huabingzhang@ahmu.edu.cn; Wei Chen, Department of General Surgery, The First Affiliated Hospital of Anhui Medical University, Hefei, Anhui, 230022, People's Republic of China, Tel +86 13966711566, Fax +86 0551-63633742, Email chenwei366@126.com; chenwei366@ahmu.edu.cn

Purpose: To evaluate the effect of 5-fluorouracil (5-FU) combined with rutaecarpine (RUT) on the antiproliferative, anti-migratory, and apoptosis-promoting ability of colorectal cancer (CRC) cells and explore the underlying mechanism.

Methods: The antiproliferative effects of RUT and 5-FU on CRC cells were evaluated using MTT and colony formation assays. Anti-migration was assessed by cell scratch and transwell tests. The synergistic effect of RUT and 5-FU was assessed by isobologram and combination index analysis using CompuSyn software. The effects of RUT and 5-FU on cell apoptosis were detected by flow cytometry. Differences in protein expression levels with or without RUT and/or 5-FU treatment were assessed by Western blot. Moreover, a mouse xenograft model of CRC was established to investigate the antitumor effect of RUT and 5-FU in vivo, and Ki67 and cleaved caspase-3 expression was detected by immunofluorescence.

Results: In this study, we found that 5-FU combined with RUT can inhibit the proliferative, migratory, and antiapoptotic abilities of CRC cells to a significantly greater extent than either RUT or 5-FU alone both in vivo and in vitro. Western blot analysis showed that the level of signal transducer and activator of transcription 3 (STAT3) phosphorylation in CRC cells was significantly reduced after combination therapy compared with that seen with the respective monotherapies. In addition, combination therapy influenced the STAT3 signaling pathway; namely, it inhibited the expression of c-Myc, CDK4, and Bcl-2 while enhancing that of the proapoptotic protein cleaved caspase-3. Immunofluorescence staining further showed that the expression of Ki67 and cleaved caspase-3 was significantly downregulated and upregulated, respectively, in tumor tissues of mice treated with combination therapy compared with that observed with monotreatment alone.

Conclusion: Combined therapy with 5-FU and RUT exerted a superior curative effect in CRC than treatment with either single drug alone and has potential as a novel therapeutic modality for the treatment of CRC.

Keywords: 5-fluorouracil, colorectal cancer, rutaecarpine, signal transducer and activator of transcription 3, synergistic therapy

Introduction

Colorectal cancer (CRC) is a malignancy that often leads to death.[1] Most patients are diagnosed in the late stage of disease development and chemotherapy is often the only treatment option. Although 5-fluorouracil (5-FU) is the most commonly used chemotherapeutic agent for CRC treatment, the modality is associated with poor overall efficacy owing to the development of drug resistance.[2] Indeed, the overall response rate of advanced CRC to this drug is only

10% to 15%.[3] Studies have shown that CRC treatment is also constrained by the toxicity associated with chemotherapeutic drugs and high resistance to treatment.[2] Despite the emergence of new chemotherapeutics, 5-FU-based regimens remain the mainstay of treatment for patients with advanced CRC.[3] 5-FU is used frequently and typically in high doses in clinical settings, and 5-FU-based treatment modalities are associated with a wide variety of adverse effects, such as constipation, diarrhea, and vomiting, among others, although these are generally manageable after treatment.[4,5]

Traditional Chinese medicine combined with chemotherapeutic drugs is used in the clinical treatment of several tumors.[6] Rutaecarpine (RUT) is a biologically active alkaloid isolated from *Cornus officinalis*, used for the treatment of headaches, abdominal pain, postpartum hemorrhage, and dysentery.[7] Numerous studies have demonstrated the potential efficacy of RUT in the treatment of diabetes, Alzheimer's disease, colitis, and other digestive disorders.[8,9] RUT can delay the development of various liver diseases and has been shown a good adjuvant effect in the treatment of different hepatic pathologies.[10] Additionally, RUT can inhibit the invasion and lung metastasis of colon cancer 26-L5 cells in vitro[11] as well as the proliferation of breast cancer cells.[12]

Natural products play a vital role in drug research and development.[13] Plants are the main source of natural drugs owing to their chemical diversity and the strong biological activity of many of their components.[14] Medicinal plants are also the source of many traditional drugs such as aspirin, quinine, paclitaxel, and digoxin.[15,16] Natural alkaloids are among the most important pharmacological agents in medicinal plants.[17] In addition, we have previously shown that RUT plays an inhibitory role in the proliferation and migration of CRC cells both in vivo and in vitro.[18] These observations suggest that the RUT has the potential for use as an adjuvant in combination with the traditional chemotherapeutic drug 5-FU in the treatment of CRC.

Notably, signal transducer and activator of transcription 3 (STAT3) signals are overactivated in CRC.[19] Several downstream mediators of STAT3 signaling—c-Myc, CDK4, Bcl-2, and cleaved caspase-3—play a crucial role in cancer cell metastasis, proliferation, and survival.[20] A major reason for 5-FU resistance is the excessive activation of the STAT3 signaling pathway.[21] Although the STAT3 pathway is regulated by various signaling mechanisms, STAT3 is stimulated by a variety of stimuli, including stress and cytokines.[22] Communication between cancer and inflammatory cells is highly dependent on STAT3 activation and interaction.[23]

In order to verify the feasibility of RUT combined with 5-FU in the treatment of colorectal cancer, we conducted in vivo and in vitro experiments. In study, we report for the first time that RUT combined with 5-FU enhances the sensitivity of CRC cells to 5-FU by downregulating STAT3.

Materials and Methods
Reagents and Antibodies

RUT (98% purity) and 5-FU (99% purity) were obtained from Aladdin, China. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), the Annexin V-FITC/PI Apoptosis Detection Kit, and dimethyl sulfoxide (DMSO) were purchased from Solarbio, Shanghai, China. The following primary antibodies were used in this study: Anti-STAT3, anti-phosphorylated (p) STAT3 (both from Cell Signaling Technologies), anti-Bcl-2, anti-c-Myc (Affinity Biosciences), anti-cleaved caspase-3 (Abclonal), anti-CDK4 (Proteintech), and anti-GAPDH (Santa). The horseradish peroxidase (HRP)-conjugated goat anti-mouse/rabbit secondary antibody used in this study was purchased from Abclonal. The electro-chemiluminescence (ECL) Western blot detection reagent was acquired from Beyotime.

Cell Culture

The colon cancer cell lines HCT116, HT29, SW480, and RKO were obtained from American Typical Culture Center. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with fetal bovine serum (FBS, Shanghai, China), penicillin (100 U/mL), and streptomycin (100 mg/mL, Shanghai, China) at 37°C under 5% CO_2.

Determination of Drug Interaction

The synergistic effect of 5-FU + RUT co-administration was investigated as described by Chou and Talalay. In brief, colorectal tumor cells were treated with different concentrations of 5-FU (10, 25, 50, 75, and 100 μM) and RUT (2.5 and 5.0 μM), and the respective IC50 values were used to select their optimum dosages. An MTT assay was used to determine cell growth viability after 24 h of treatment, and then the combination index (CI) was computed using CompuSyn software version 1.0 (ComboSyn, Paramus, NJ, USA) to define the effect categories. Antagonistic, additive, and synergistic effects were denoted by CI >1, CI = 1, and CI <1, respectively.[24]

MTT Assay

CRC cells were seeded in 96-well plates at a density of 2×10^3 cells per well and allowed to adhere. The cells were then treated either with different concentrations of the respective drugs or a similar quantity of DMSO for 24 h and then incubated for 1 h at 37°C with 25 μL of MTT solution. After aspirating the medium, each well was filled with 100 μL of DMSO, followed by a 10-min incubation. Finally, the absorbance of each well at a wavelength of 490 nm was measured using a microplate reader.

Colony Formation Assay

Cells were seeded in 6-well plates at a density of 1000 cells per well, allowed to adhere to the plates, and then treated with 5 μM RUT, 10 μM 5-FU, or 5 μM RUT + 10 μM 5-FU. Cells in the control group were treated with an equal volume of DMSO. The medium was aspirated out every 3 days and replaced with fresh DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin, and processed according to the above design. After 12 days of incubation, the cells were fixed in methanol, washed twice with PBS, dried at room temperature, and stained with 0.1% crystal violet at room temperature. Finally, the number of colonies was counted.

Wound Healing Assay

First, horizontal lines were evenly drawn on the bottom of 6-well plates using a pen. CRC cells were then uniformly seeded in the marked plates at a density of 5×10^5 cells per well, allowed to adhere, and then incubated with DMSO (control), 5 μM RUT, 10 μM 5-FU, or 5 μM RUT + 10 μM 5-FU at 37°C with 5% CO_2. Scratches were made in the monolayer when the cells were close to confluence, and cell migration was recorded at 0, 24, and 48 h under an inverted microscope. Changes in wound width were analyzed using ImageJ software.

Transwell Assay

Approximately 5×10^4 CRC cells were suspended in 200 μL of serum-free medium containing 5 μM RUT, 10 μM 5-FU, or DMSO and separately added to the upper chamber of Transwell inserts. The lower chamber was filled with 500 μL of culture medium supplemented with 20% FBS. After 24 h of incubation at 37°C, cells that had crossed the membrane were fixed in 4% formaldehyde at room temperature for 20 min, washed twice with PBS, soaked for 30 min in 0.1% crystal violet at room temperature, and finally counted under an inverted microscope.

In vitro Determination of Apoptosis

CRC cells were uniformly seeded into 6-well plates at a density of 5×10^5 cells per well, allowed to adhere, and then incubated with DMSO, RUT (5 μM), 5-FU (10 μM), or their combination for 24 h. Once the medium had been removed, the cells were washed three times with warm PBS and digested with trypsin without EDTA. After washing twice with cold PBS, the cells were incubated first with annexin V binding buffer, then with annexin V-FITC dye for 15-min in the dark at 4°C, and finally with propidium iodide for 5 min in the dark at 4°C. Cell apoptosis was detected by flow cytometry.

Western Blot

Total protein was extracted from cells using RIPA lysis buffer (Beyotime). Equal concentrations of protein from each group were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred to PVDF membranes. The membranes were blocked with 5% skimmed milk for 1 h at room temperature, incubated overnight at 4°C with primary antibodies targeting c-Myc (1:1000), CDK-4 (1:2000), Bcl-2 (1:1000), GAPDH (1:1000), cleaved caspase-3 (1:1000), STAT3 (1:2000), or p-STAT3 (1:1000), washed, and incubated for 1 h with HRP-coupled secondary antibodies at room temperature. The protein bands were detected using ECL reagent.

Determination of Antitumor Activity in vivo

Four-week-old female BALB/c mice, weighing 15 to 20 g, were acquired from the Model Animal Research Centre of Nanjing University and kept under specific pathogen-free conditions. All experimental procedures involving animals were conducted in accordance with the guidelines of the Animal Experimentation Center of Anhui Medical University and strictly adhered to China's Law on the Use and Care of Laboratory Animals. The experimental protocols were approved by the Animal Ethics Committee of Anhui Medical University. Each mouse was subcutaneously injected with 1×10^6 of an HCT cell suspension near the right axilla and the tumor size and weight of each mouse were measured every other day. The maximum longitudinal and transverse diameter of each tumor was also measured using calipers and the total tumor volume was calculated as (length×width^2)/2. When the tumor volume approached 50-100 mm^3, mice were randomly distributed into the following groups and administered the indicated treatments every 2 days: Saline (control), RUT (30 mg/kg), 5-FU (10 mg/kg), or RUT (30 mg/kg) + 5-FU (10 mg/kg). The experiment lasted for a total of 20 days, with 10 intraperitoneal injections administered to each treatment group. Tumor weights and volumes in each group were measured on day 20 after treatment, following which the animals were euthanized.

Immunofluorescence

Tumor tissues were embedded in paraffin, dehydrated in a graded ethanol series, cut into 5-mm-thick slices, and fixed at room temperature in 10% neutral-buffered formalin. For immunostaining, antigen retrieval was performed with citric acid (pH 6.0) followed by treatment with 3.0% H_2O_2 in methanol to eliminate the effect of endogenous peroxidases. The sections were incubated overnight with primary antibodies targeting Ki67 or cleaved Caspase 3 at 4°C, washed, and then incubated with biotinylated secondary antibodies for 1 h at room temperature. The sections were finally visualized and imaged under an immunofluorescence microscope.

Statistical Analysis

All data were analyzed using GraphPad Prism version 9.0 (GraphPad Software, La Jolla, CA, USA) and are presented as means ± standard deviation (SD). Variables across multiple groups were compared using one-way analysis of variance (ANOVA) followed by Tukey's HSD test. Each experiment was performed three times. *P*-values <0.05 were considered significant.

Results
Rut/5-FU Inhibits Cell Survival

The chemical structures of 5-FU and RUT are shown in Figure 1A and B. We first treated HCT116, HT29 and SW480 cells with different concentrations of RUT or 5-FU for 24 h, and then determined the effect on cell survival using the MTT assay. Similarly, RKO cells were treated with RUT and/or 5-FU at different concentrations for 24 h and then subjected to the MTT assay. No significant differences in cell survival were detected (Figure S1A–D). Both RUT and 5-FU exerted an inhibitory effect on the survival of three CRC cell types in a dose-dependent manner, although the effect tended to level off. According to the fitting formula, after 24 h of treatment, the IC50 values of RUT for HCT116, HT29, and SW480 cells were 24.74, 21.48, and 17.79 μM, respectively. For 5-FU, the respective IC50 values were 74.47, 85.14, and 69.14 μM (Figure 1C–H). These results suggested that the therapeutic effect of each drug could not be achieved by simply increasing the concentrations of the drugs. Notably, 24 h of treatment with 5-FU at a concentration of 10 μM or
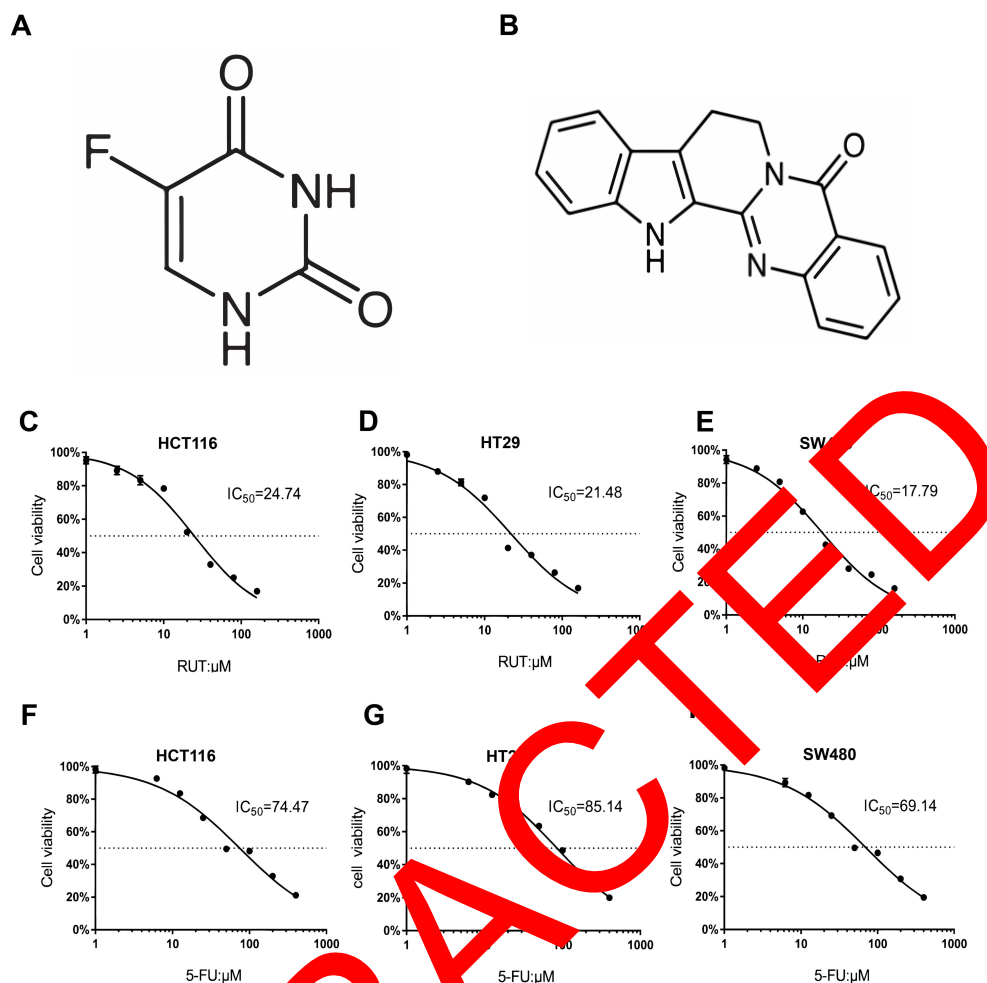
Figure I RUT, 5-FU inhibited proliferation of HCT116, HT29 and SW480 cells. (**A** and **B**) Chemical structure of 5-FU and RUT. (**C–H**) The cell viability rate analysis of RUT (2.5, 5, 10, 20, 40, 80, 160 μM) or 5-FU (6.25, 12.5, 25, ~, 100, 200, 400 μM) treatment. The 24 h IC50 in 3 cell lines were shown respectively.

RUT at a concentration of 5 μM did not significantly affect cell viability. Consequently, we selected 10 μM and 5 μM as the optimum concentrations for 5-FU and RUT, respectively, for use in subsequent experiments.

Table 1 summarizes the combined index (CI) values for RUT and 5-FU at different concentrations on HCT116, HT29, and SW480 cells. CI values less than 1 indicated the presence of synergy. Specifically, CI values from 0.1~0.3 indicated strong synergy, values from 0.3~0.7 indicated synergy, those from 0.7~0.85 indicated medium synergy, and those from 0.85~0.9 indicated weak synergy. In Table 1, the CI values for all the RUT and 5-FU combinations were lower than 0.3, indicating that RUT and 5-FU exerted a strong synergetic effect on the proliferation of HCT116, HT29, and SW480 cells

Table I The Combination Index (CI) Values of Combinations of RUT with 5-FU in Three Cell Lines

Combinations		CI		
Dose RUT:μM	Dose 5-FU:μM	SW480	HCT116	HT29
2.5	10	0.1609	0.1918	0.1141
2.5	25	0.2043	0.1982	0.1819
2.5	50	0.1849	0.2197	0.1673
2.5	75	0.1512	0.1816	0.1853

(*Continued*)

Table 1 (Continued).

Combinations		CI		
Dose RUT:μM	Dose 5-FU:μM	SW480	HCT116	HT29
2.5	100	0.1404	0.1902	0.1418
5	10	0.1487	0.1077	0.1090
5	25	0.1311	0.0824	0.1246
5	50	0.1584	0.0809	0.1198
5	75	0.0952	0.0716	0.0860
5	100	0.0942	0.0728	0.0848

Notes: CI Data for Non-Constant Combo: 5-FU+RUT.
Abbreviations: RUT, Rutaecarpine; 5-FU, 5-fluorouracil.

within the evaluated concentration range. Notably, the drug combination regimen significantly inhibited cell proliferation compared with the respective monotherapies (Figure 2A–F). The results of the isobologram and combination index analysis are shown in Figure 3A–F. A colony formation assay further confirmed the inhibitory effect of the drug
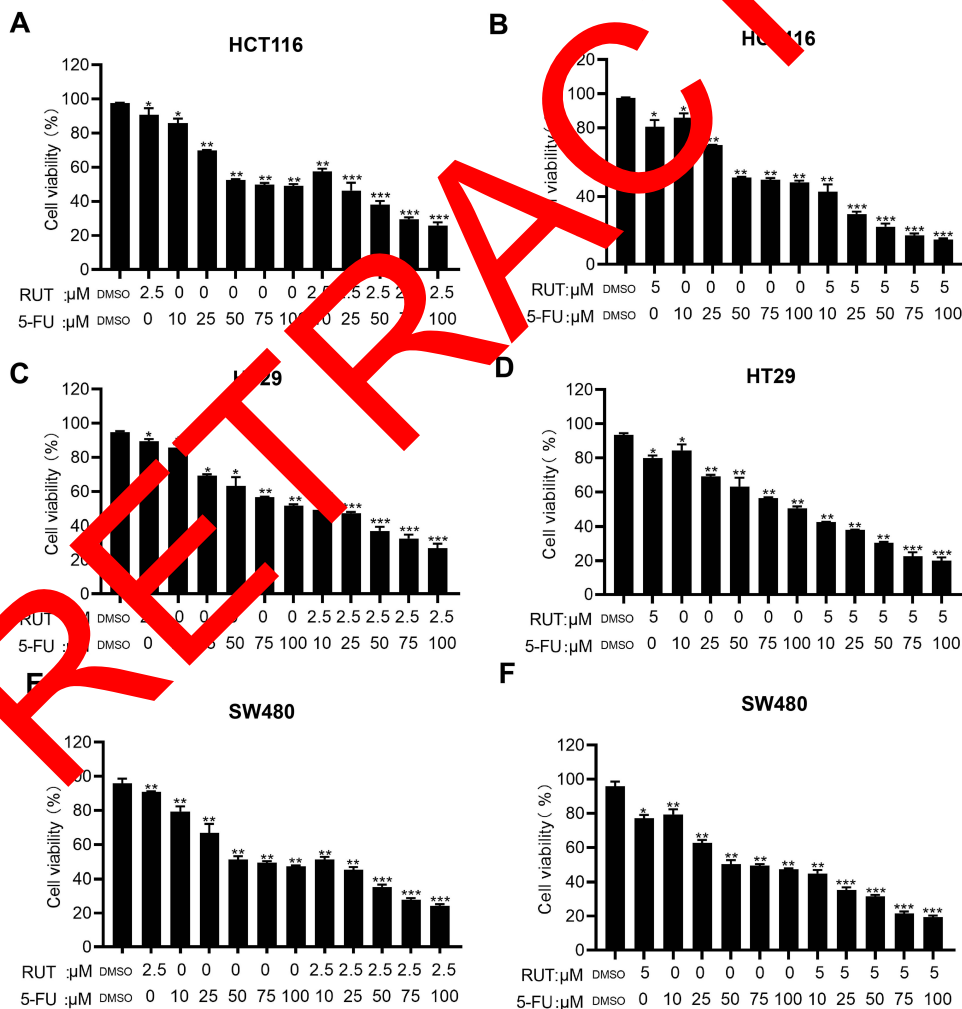

Figure 2 (**A–F**) MTT assay was used to evaluate cell viability rate of colorectal tumor cells (HCT116, HT29 and SW480) treated with DMSO, RUT (2.5, 5μM) and or 5-FU (10, 25, 50, 75, 100μM) for 24 h alone or in combination. n=3,*P < 0.05, **P < 0.01, ***P < 0.001 compared with DMSO group.

combination on tumor cells (Figure 3G–L). Collectively, these results demonstrated that RUT and 5-FU synergistically interact in enhancing the cytotoxicity of 5-FU against various types of CRC cells.

RUT/5-FU Inhibits Cell Migration

To elucidate the effect of RUT (5 μM) + 5-FU (10 μM) on the migratory capacity of CRC cells, we next performed wound healing and Transwell assays. The results revealed that cells treated with the combination therapy migrated significantly shorter distances compared with the controls. In addition, CRC cells in the combination treatment group displayed markedly reduced migration capacity compared with those in the individual RUT and 5-FU treatment groups
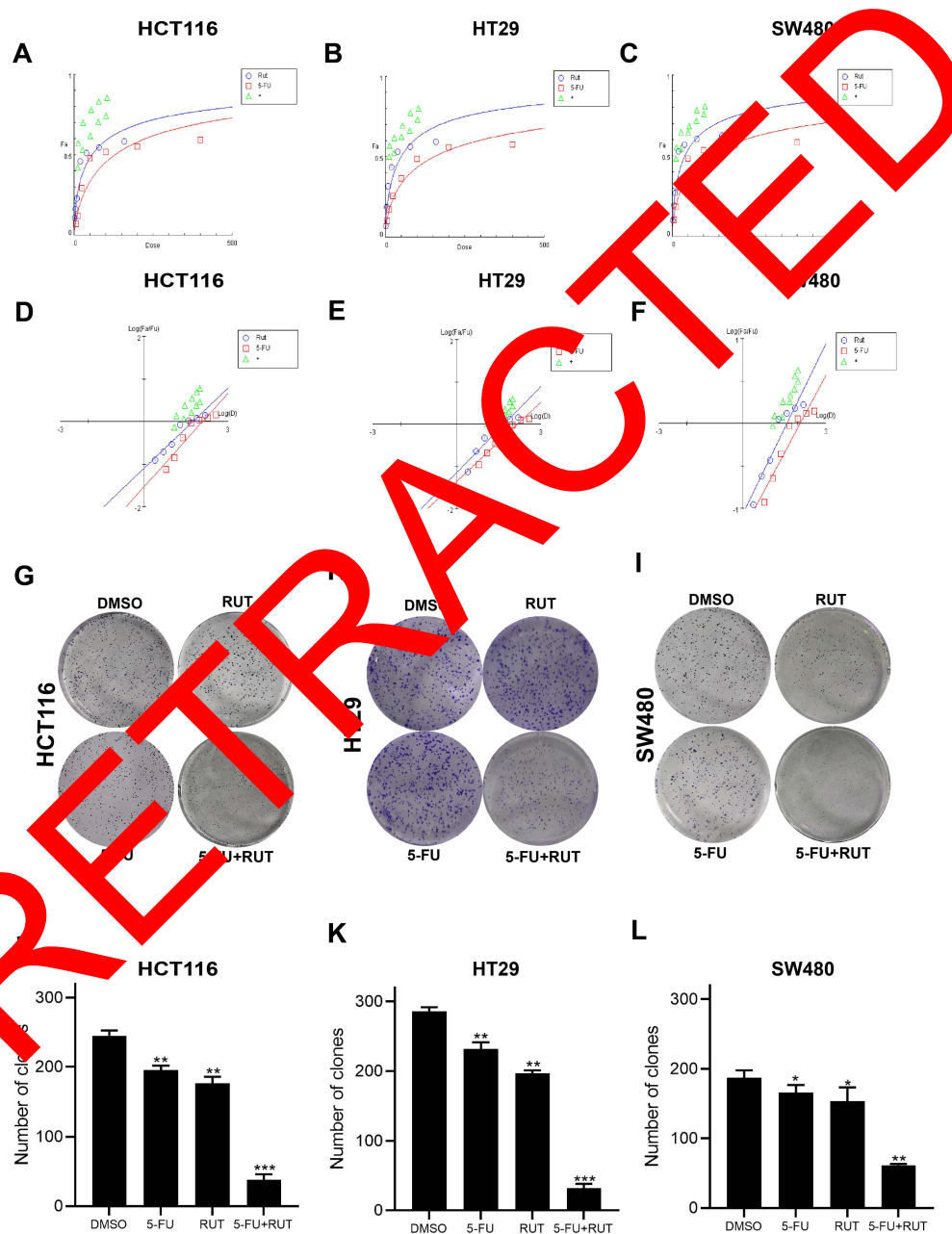

Figure 3 (**A–F**) The synergistic effects of the combination of RUT (2.5, 5μM) and 5-FU (10, 25, 50, 75, 100μM) for 24 h were analyzed by dose effect diagram and medium effect diagram, respectively. (**G–I**) The effect of DMSO (control), RUT (5μM), 5-FU (10μM), RUT (5μM) and 5-FU (10μM) on colony formation. (**J–L**) Bar graphs represent means ± standard deviation (SD) of colonies in HCT116, HT29, and SW480 cells treated with 5μM RUT with or without 10μM 5-FU for 12 days. n=3,*P < 0.05, **P < 0.01, ***P < 0.001 compared with DMSO group.

(Figure 4A–F). Collectively, these results indicated that 5-FU combined with RUT significantly inhibited CRC cell migration in vitro compared with the individual monotherapies (Figure 4G–L).

RUT and 5-FU Interact Synergistically to Induce Apoptosis in CRC Cells

The results of the apoptosis assay showed that individual RUT (5 μM) and 5-FU (10 μM) treatments increased the rates of cell apoptosis relative to the control condition, although the effect was weak. After 24 h of treatment, RUT or 5-FU
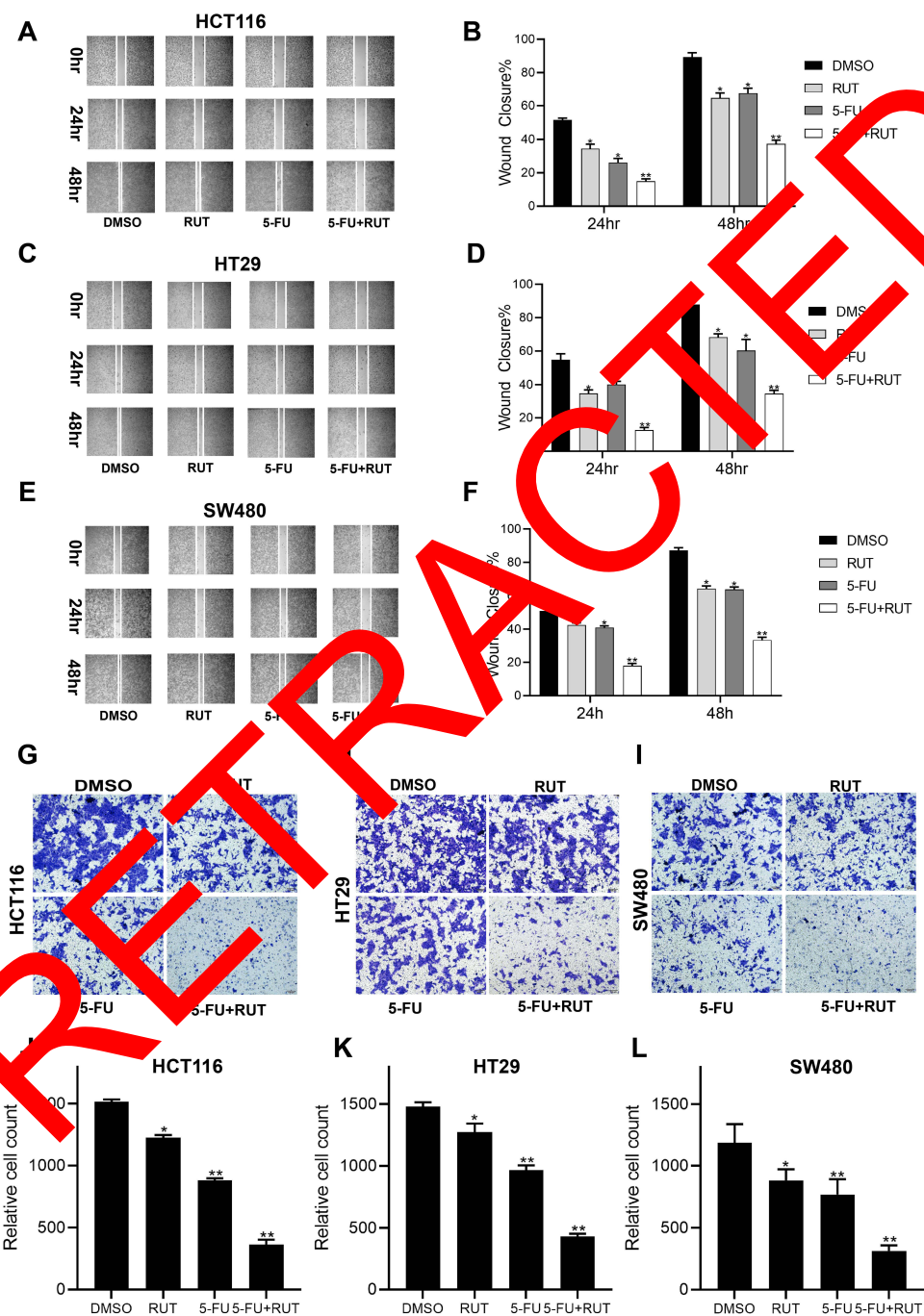

Figure 4 Wound and Transwell assays results showing the effects of different drug treatments on migration of HCT116, HT29 and SW480 cell. (**A–F**) The results of wound healing test showed that the migration ability of cells treated with DMSO (control), RUT (5μM), 5-FU (10μM), RUT (5μM) and 5-FU (10μM). (**G–L**) The results of transwell assay showed that the migration ability of cells treated with DMSO (control), RUT (5μM), 5-FU (10μM), RUT (5μM) and 5-FU (10μM). (Scale bar: 100μm). n=3,*P < 0.05, **P < 0.01, compared with DMSO group.

treatment alone did not lead to significant changes in the number of apoptotic cells in HCT116, HT29, or SW480 cells. Notably, combined treatment with 5 μM RUT + 10 μM 5-FU had a significant effect on the rate of apoptosis and enhanced the apoptotic effect of 5-FU, which were 26.85% in HCT116 cells, 24.8% in HT29 cells, and 28.93% in SW480 cells (Figure 5A–F). Combination therapy resulted in a significantly higher rate of cell apoptosis relative to the respective individual treatments. Taken together, these findings demonstrated that, compared with the respective mono-therapies, 5-FU + RUT combination treatment significantly increases the rate of apoptosis in CRC cells in vitro.

RUT Enhances the 5-FU-Mediated Inhibitory Effect on the STAT3 Signaling Pathway

The STAT3 signaling pathway plays an important role in the proliferation, progression, and chemoresistance of CRC. In the present study, we quantified the levels of STAT3 expression by Western blot (Figure 5G–N). In all three CRC cell
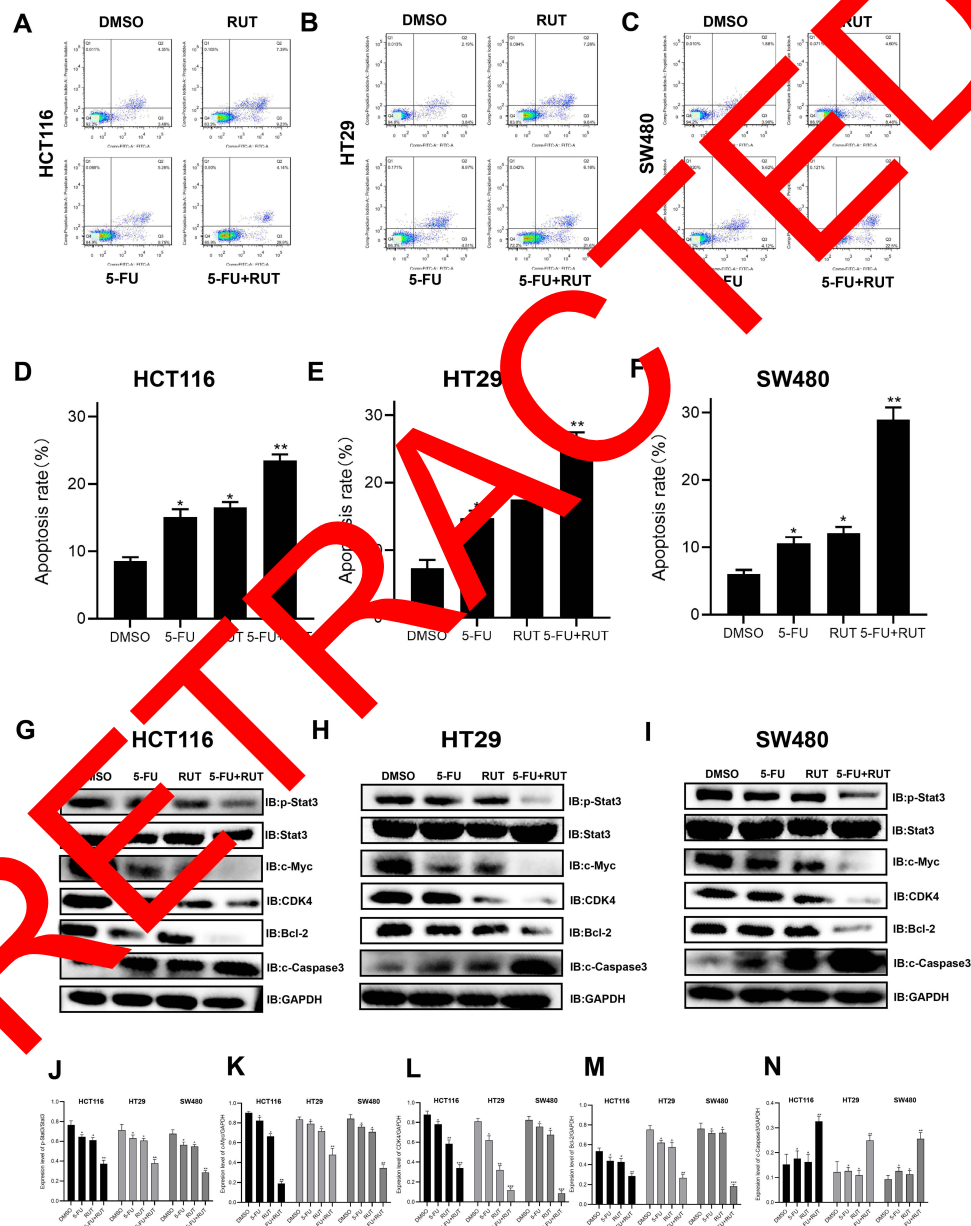

Figure 5 (**A–F**) Apoptosis-inducing effect of RUT, 5-FU and their combination on HCT116, HT29 and SW480 cells. The percentage of apoptotic cells was determined by flow cytometry after being treated with DMSO, RUT, 5-FU and their combination respectively for 24 h. (**G–N**) The expression of related proteins (p-STAT3, STAT3, c-Myc, GAPDH, CDK4, Bcl-2, c-Caspase 3) in cells treated with DMSO, RUT, 5-FU and 5-FU+RUT were detected 24 h later. n=3,*P < 0.05, **P < 0.01, ***P < 0.001 compared with DMSO group.

lines, the combination therapy significantly inhibited STAT3 phosphorylation. Consequently, STAT3 expression was significantly lower in tumors of the combination treatment group than in those treated with RUT or 5-FU alone. Next, we analyzed the expression of CDK4 and c-Myc, both of which are regulated by STAT3 and have been shown to play a role in tumor cell proliferation and apoptosis; as well as that of Bcl-2 and cleaved caspase-3, the overexpression of which has been strongly correlated with tumor survival and resistance to chemotherapy. Western blot results indicated that, in contrast to monotherapy, RUT + 5-FU treatment significantly downregulated the expression of all these proteins in CRC
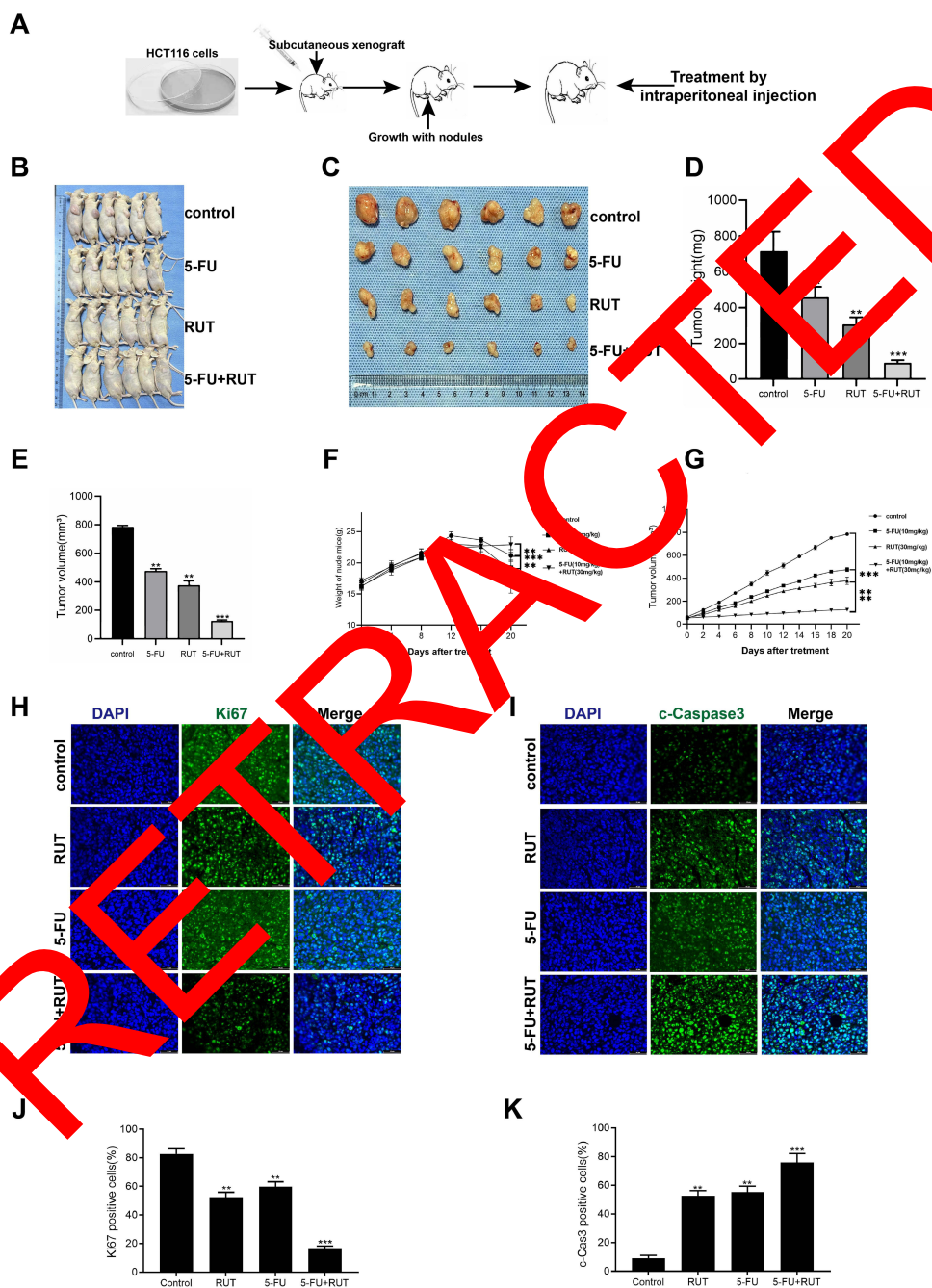

Figure 6 In vivo antitumor effects of RUT and or 5-FU on transplanted HCT116 cells in nude mice. (**A**) procedure for establishment of a xenograft model and injection of normal saline (control), RUT (30mg/kg), 5-FU (10mg/kg) and RUT (30mg/kg) + 5-FU (10mg/kg) for 20 days. (**B** and **C**) tumor images. (**D**) Tumor weight at the end of the experiment. (**E**) Tumor volume at the end of the experiment. (**F**) Weight changes in nude mice in different treatment groups. (**G**) volume changes in nude mice were recorded every two days during the whole treatment period. (**H–K**) H&E staining and statistical results of tumor tissue in groups. (Scale bar: 50μm). n=6, *P < 0.05, **P < 0.01, ***P < 0.001, compared with the control group.

tumor tissues. Collectively, these findings suggested that RUT + 5-FU synergistically interact to inhibit STAT3 and STAT3-regulated protein expression.

RUT + 5-FU Therapy Plays an Anticancer Role in vivo

Next, we established a human CRC (HCT116 cells) xenograft model in BALB/c nude mice and evaluated the synergistic effects of RUT and 5-FU in vivo. Schematic representation of the animal experimentation process is illustrated in Figure 6A. We found that the combination therapy markedly suppressed tumor volume and weight compared to 5-FU monotherapy. Analysis of tumor images 20 days after treatment revealed significant differences ($p<0.05$) in tumor volume (Figure 6B). Treatment with RUT (30 mg/kg) or 5-FU (10 mg/kg) alone reduced tumor growth by respectively 32.9% and 35.8% (Figure 6C and D) and tumor weight by respectively 33.7% and 63.4% (Figure 6E–G) after 20 days of treatment relative to that seen in the control (saline treatment) group. Notably, RUT + 5-FU treatment significantly inhibited tumor growth ($p<0.05$), reducing tumor weight and volume by 90% and nearly 90%, respectively. These results suggested that RUT + 5-FU combination therapy exerts a synergistic antitumor effect in vivo. In addition, compared with the controls, the number of Ki67-positive cells was decreased and the percentage of cleaved caspase-3-positive cells was significantly increased in tumor tissues of the combination therapy group. In conclusion, our findings indicated that the drug combination inhibited the proliferative ability of CRC cells in vivo by upregulating the expression of cleaved caspase-3 and downregulating that of Ki67, there were statistical differences between all groups (Figure 6H–K).

Discussion

Lifestyle, genetic, and environmental factors have all been shown to be risk factors for the development of CRC.[25] This malignancy is the third most common type of cancer worldwide. It is associated with a high prevalence in the West as well as rising mortality rates in China.[26,27] Research efforts to date have focused on the development of agents that can effectively kill cancer cells with minimal side effects. Currently, surgery, radiation, and chemotherapy are applied in cancer treatment, although each therapy is associated with drawbacks.[28] Typically, the efficacy of chemotherapy modalities is limited by the resistance of the diseased tissues and the toxicity to healthy ones.[29]

In recent years, the combination of natural plant active ingredients and traditional chemotherapy drugs has attracted more and more attention.[30,31] Combined therapy can enhance synergy and reduce the occurrence of chemotherapy drug resistance and adverse reactions, which is particularly important for anti-tumor effect.[32,33] This enhanced therapeutic effect can reduce the dose required for each drug alone, which may alleviate the potential adverse reactions associated with long-term high-dose administration of single drugs. Rutaecarpine, a promising natural compound, has attracted more and more attention, especially in its potential anticancer effect.[34,35] In previous study, we found that RUT could inhibit cell proliferation and migration, and promote cell apoptosis of CRC cells by regulating STAT3 signal pathway.[18] A large number of studies also show that the effective ingredients of various plants can enhance the therapeutic effect of 5-FU. For example, curcumin enhances 5-FU sensitivity by increasing ROS and cell cycle arrest in colorectal cancer cells.[36] Quercetin targets Wnt/β-The catenin signaling pathway enhances the effect of 5-fluorouracil on human colon cancer cells.[37] Coptis chinensis extract reduces 5-fluorouracil resistance by regulating thymidylate synthase.[38] Tenacissoside G enhances the inhibitory effect of 5-fluorouracil on human colorectal cancer by blocking cell cycle progression and inducing p53-mediated apoptosis.[24] Therefore, we envisage to use RUT as an adjuvant in combination with traditional chemotherapy drug 5-FU, and carry out experimental verification.

In the current study, we found that the combination treatment has a significant effect. At the RUT and 5-FU concentrations of 5 and 10 μM, respectively, the individual drugs exerted only a limited inhibitory effect on the proliferation of CRC cells. However, when the two drugs were administered in combination, a significant tumor-suppressive effect was observed, even at low concentrations. In vivo, at low concentrations, neither 5-FU nor RUT significantly delayed tumor growth or metastasis when administered alone. However, RUT + 5-FU combination therapy significantly inhibited tumor growth and metastasis while also increasing sensitivity to 5-FU. To further evaluate the synergistic effect of the two drugs, we employed an isobologram and combination index analysis. The results showed that the two drugs have a strong synergistic effect. We found that RUT significantly promoted the chemosensitivity of CRC cells to 5-FU, both in vivo and in vitro, whereas no additive effect of 5-FU on CRC cell proliferation was observed

with increasing concentrations of 5-FU, even at very high concentrations. We used Western blot assays to determine the levels of p-STAT3 and STAT3 in SW480, HCT116, and HT29 cells, and then applied immunohistochemistry to evaluate Ki67 and cleaved caspase-3 expression in tumor tissues under the different treatments. We found that neither RUT nor 5-FU alone significantly inhibited the STAT3 pathway, whereas RUT + 5-FU combination therapy significantly downregulated STAT3 expression in CRC cells. Studies have shown that blocking the STAT3 signaling pathway can restore sensitivity to 5-FU.[39] Additionally, STAT3 is overactivated in many tumors and plays a key role in the proliferation and survival of tumor cells and their metastasis, as well as in chemotherapy resistance.[40,41] Accordingly, we attribute the above results to the synergistic effect of RUT and 5-FU on the inhibition of STAT3 activity.

We further found that the STAT3-regulated antiapoptotic protein Bcl-2 and the proliferative proteins c-Myc and CDK4 were significantly downregulated after healing, which is consistent with results from previous studies. These findings suggested that RUT inhibits STAT3 and STAT3-regulated antiapoptotic as well as antiproliferative proteins, thereby enhancing 5-FU-mediated antitumor effects. In addition, our findings demonstrated that 5-FU and RUT not only upregulated the expression of caspase-3, a proapoptotic protein but also concomitantly inhibited that of Ki67, a marker of proliferation. These observations indicate that CRC cells are likely to become more receptive to 5-FU due to the synergistic inhibitory effects on STAT3 signaling resulting from RUT + 5-FU combination therapy.

Cancers, such as CRC, have been associated with elevated STAT3 activity. Here, we observed that STAT3 expression was correlated with 5-FU resistance in CRC cell lines. This result is of wide clinical significance given that most rectal cancers are resistant to preoperative multimodal therapy.[43] Moreover, STAT3 protein expression has also been linked with increased drug resistance. For example, in resistant cell lines, the levels of phosphorylated STAT3 were significantly enhanced following interleukin 6 (IL-6) activation, even though STAT3 had no constitutive activity. Another study demonstrated that STAT3 is a promising molecular marker with a potential role to mediate drug resistance.[18]

Studies have shown that the inflammatory mediator IL-6 triggers the expression of the gene that stops tumor cell cycle progression and apoptosis. Moreover, this gene plays an important role not only in epithelial–mesenchymal transition (EMT) in tumor cells but also in the regulation of immune and inflammatory responses. Interestingly, IL-6 binds to receptor/glycoprotein 130 (gp130) and upregulates the expression of the Janus kinase (JAK)/STAT3 signaling pathway in CRC cells, thereby promoting EMT and resistance to chemotherapy.[44]

At present, multidrug combination therapy is widely used to reduce resistance to chemotherapy and improve its efficacy. Although taking 5-FU in conjunction with other medications such as FOLFOX and FOLFIRI was reported to slow CRC progression to some extent, these modalities were also associated with significant toxicity. Consequently, there is an urgent need to identify approaches to improve the efficacy of 5-FU without exacerbating the associated side effects.[45]

Conclusion

In summary, we report for the first time the synergistic anticancer effect of RUT and 5-FU on human CRC cells (HCT116, SW480, and HT29), findings that were validated in a xenograft mouse model. We attribute this effect to the downregulation of STAT3 phosphorylation. Taken together, our findings demonstrated that the 5-FU + RUT combination has potential as a therapeutic modality for the treatment of CRC.

Abbreviations

CRC, colorectal cancer; 5-FU, 5-fluorouracil; RUT, rutaecarpine; STAT3, signal transducer and activator of transcription 3; Bcl-2, B-cell lymphoma-2.

Data Sharing Statement

Data supporting these findings can be obtained from the corresponding authors upon reasonable request.

Ethics Approval

No human subjects were involved in this study. All animal experiments were carried out in strict accordance with the guidelines of the Animal Center of Anhui Medical University. Experimental procedures were approved by the Animal Ethics Committee of Anhui Medical University.

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Author Contributions

All the authors made a significant contribution to the study, whether in its conception, design, execution, acquisition of data, analysis, and interpretation, or all these areas. Additionally, all the authors participated in drafting, revising, or critically reviewing the article; gave approval for the final version to be published; agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work. Wei Chen and Huabing Zhang supervised the study.

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Disclosure

The authors report no conflicts of interest in this work.

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