Investigating the Pharmacological Mechanism of Zhengyuan Jiaonang for Treating Colorectal Cancer via Network Pharmacology Analysis and Experimental Verification

Haidong Denga,1, Siqi Liua,1, Didi Lib, Weiping Wangc, Ling Yea, Shaofeng Xuc, Xiaoliang Wangc,a, Yan Lib,*

a Beijing Key Laboratory of New Drug Mechanisms and Pharmacological Evaluation Study, Department of Pharmacology, Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100050, China
b Thousand Dimensions (Beijing) Science and Technology Co., Ltd, Beijing 102699, China
c State Key laboratory of Bioactive Substances and Functions of Natural Medicines, Department of Pharmacology, Institute of materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100050, China

* Correspondence: liyanxiao@imm.ac.cn, +8613641058345 (Yan Li), wangxl@imm.ac.cn, +8613701166880 (Xiaoliang Wang);

1 Haidong Deng and Siqi Liu contributed equally to this article.

Highlights
Chemical constituents of Zhengyuan Jiaonang (ZYJN) was identified with fingerprint analysis.
The antitumor effects of ZYJN in subcutaneous transplant CT26 model were investigated.
The effects of ZYJN on the landscape of tumor-infiltrating immune cells in CT26 model were investigated by flow cytometry.
Reveal how ZYJN affects tumor fibrosis and blood vessels by transparent imaging analysis.

Abstract
Ethnopharmacological relevance
Zhengyuan jiaonang (ZYJN) is a traditional Chinese patent medicine (CPM) used in China for adjuvant cancer therapy, which has been proved to have anti-fatigue effects.

Aim of study
The study aims to investigate the antitumor effects of ZYJN and its underlying mechanisms using subcutaneous transplant CT26 model.

Materials and methods
Fingerprint analysis of ZYJN was performed using high performance liquid chromatography. The potential targets of ZYJN were predicted using bioinformatic analysis, which were further validated by Western Blot assay. Subcutaneous transplant CT26 model was used to evaluate the antitumor effects of ZYJN. The effects of ZYJN on the tumor immune microenvironment were investigated by flow...
Cytometry. Transparent imaging was used to investigate the effects of ZYJN on fibrosis and angiogenesis.

Results

ZYJN could inhibit colorectal cancer growth when administered alone or in combination with 5-FU. The combination of ZYJN and 5-FU could significantly increase the serum level of albumin (ALB) and decrease the serum level of aspartate aminotransferase (AST). In addition, the combination of ZYJN at 0.75 g/kg and 5-FU significantly decreased the serum level of vascular endothelial growth factors (VEGF) and inhibited the angiogenesis of CT26 cancer. The combination of ZYJN at 1.50 g/kg and 5-FU could promote the fibrosis process of CT26 cancer. Additionally, combination of ZYJN and 5-FU could significantly increase the percentage of tumor-infiltrating T cells and CD4$^+$ T cells in the late stage of CT26 model, while ZYJN at 1.50 g/kg increased the percentage of NK cells as well as CD8$^+$ T cells in the early stage of CT26 model. Western Blot analysis revealed that administration of ZYJN at 0.75 g/kg reduced the expression of PI3K-p110α, CDK1, CCNB1 and MMP-9, and inhibited the phosphorylation of Akt (Thr308).

Conclusions

ZYJN could inhibit the tumor growth of CT26 colorectal cancer by promoting tumor fibrosis, suppressing angiogenesis, migration, and invasion and modulating the tumor immune microenvironment. ZYJN enhanced the efficacy and reduced the toxicity of chemotherapy drugs in combination therapy. Our findings provide evidence for the clinical application of ZYJN in cancer treatment.

Key words: Colorectal cancer; Traditional Chinese medicine; Antineoplastic; Zhengyuan Jiaonang.

1. Introduction

Colorectal cancer (CRC) is the second leading cause of cancer death in the United States and the third most common cancer worldwide (Morgan et al., 2023). Although the incidence has decreased over the past 10 years, the incidence of CRC has tended to be in younger individuals and diagnosed at a later stage (Siegel et al., 2023). 5-Fluorouracil (5-FU) is a commonly used drug for chemotherapy and combined radiotherapy (Piawah and Venook, 2019), and its oral prodrug capecitabine has similar efficacy. In recent years, combination regimens such as oxaliplatin, bevacizumab and cetuximab have been proposed as alternatives (Brenner et al., 2014). For nonmetastatic colorectal cancer, surgery is the preferred option; for metastatic cancer, the combination of chemotherapy agents and the use of targeted agents may be the first choice for patients (Kuipers et al., 2015). Despite the availability of various treatments for colorectal cancer, the mortality rate is still high, and the quality of life of patients is severely compromised. Therefore, more effective therapies are still urgently needed.

Traditional Chinese medicine has been widely used in China for a long time, but it is difficult to popularize and recognize due to the lack of understanding of its chemical composition and pharmacological effects. In recent years, the therapeutic effects of certain traditional Chinese medicines on cancer treatment have been verified, with few side effects. It affects the occurrence and development of tumors through multiple links and targets and can play a certain role in the treatment of tumors by regulating...
the immune function of patients. At present, the exploration of the effective 
ingredients and pharmacological effects in traditional Chinese medicine formulas has 
gradually drawn the attention of researchers. Zhengyuan jiaonang (ZYJN) is an oral 
Chinese medicine preparation used in the adjuvant treatment of lung cancer in China, 
with validated anti-fatigue effects and ability to improve the efficacy of chemotherapy. 
(Zhang et al., 2021). The main ingredients of ZYJN are *Epimedium sagittatum* (Sieb. 
et Zucc.) Maxim (common name: Yin-yang-huo in China), *Panax ginseng* C.A. Mey 
(common name: Ren-shen in China), *Astragalus mongholicus* Bunge (common name: 
Huang-qi in China), *Ligustrum lucidum* Ait (common name: Nv-zhen-zi in China), 
*Atractylodes macrocephala* Koidz (common name: Bai-zhu in China), *Citrus 
reticulata* Blanco (common name: Chen-pi in China) and turtle shell powder 
(common name: Gui-jia in China) (The herbal names have been checked with MPNS 
(http://mpns.kew.org)). This study aims to analyze and confirm the active components 
of ZYJN with TCM network pharmacology and fingerprint analysis and explore its 
efficacy and mechanism in the treatment of colorectal cancer and adjuvant therapy.

2. Materials and methods

2.1 Cell lines and culture

The mouse colorectal cancer cell line CT26 was acquired from Nanjing Cobioer 
biosciences co., LTD. CT26 cells were cultured in Roswell Park Memorial Institute 
1640 Medium (RPMI 1640 Medium, Gibco, China) supplemented with 10% (v/v) 
fetal bovine serum (Beijing YuanHeng ShengMa Biology Technology Research 
Institute, China), 100 U/mL of penicillin, and 100 μg/mL of streptomycinand 
(Beyotime Biotechnology, China) in a 37°C humidified incubator containing 5% CO₂.

2.2 Drugs and compounds

ZYJN was acquired from Yangtze River Pharmaceutical Group Guangzhou Hairong 
Pharmaceutical Co., Ltd. Batch number of ZYJN used in animal experiments 
51201018, 51201101. Batch number of ZYJN used for fingerprint analysis: 51191234, 
51191235, 51191236, 51191237, 51191239, 51191241, 51191244, 51200102, 
51200105, 51200107.

2.3 Fingerprint analysis of ZYJN

To obtain a stock solution of ZYJN, 1.0 g dry powder was dissolved in 10 mL 
methanol, and ultrasonic treatment (power 500 W, frequency 40 kHz) was performed 
for 30 min. After cooling, methanol was used to compensate for the weight lost. Then 
filtered the solution through a 0.45 μm filter membrane for subsequent use. The 
fingerprint analysis of ZYJN was conducted on a high performance liquid 
chromatography (HPLC, Waters e2695, USA) instrument equipped with a diode array 
detector and an Empower chromatography work station. A Welch Ultimate XB-C18 
column (4.6 × 250 mm, 5 μm) was used to separate the various constituents in ZYJN. 
Acetonitrile (solvent B) and pure water (solvent D) made up the mobile phase. The 
following gradient was used for elution: 0–30 min, 15%-26% B; 30–45 min, 26% B; 
45–70 min, 26%-54% B; 70–75 min, 54%-90% B; 75-90 min, 90%. The injection 
volume was 10 μL, and the column temperature was 30°C. The samples were 
identified at 203 nm (flow rate: 1.0 mL/min).
2.4 Traditional Chinese Medicine Systems Pharmacology Database and Analysis (TCMSP)

The herb sources of ZYJN ingredients were identified by TCMSP (Ru et al., 2014). TCMSP is a unique web platform for Chinese herbal medicine and can be used to investigate the association of drug-targets-disease as well as the pharmacokinetic properties of natural compounds.

2.5 Swiss target prediction

The potential targets of 12 ingredients were searched in SwissTargetPrediction website (http://www.swisstargetprediction.ch), which allows users to predict putative biotargets of inputted small molecules (Daina et al., 2019).

2.6 Protein–protein interaction (PPI) network construction

STRING (https://cn.string-db.org/, version 11.5) is a web set from the infrastructural part of ELIXIR for protein–protein interaction (PPI) networks and functional enrichment analysis (Szklarczyk et al., 2019). It includes direct (physical) and indirect (functional) interactions. The PPIs of putative targets were predicted using STRING. The minimum required interaction score was set as 0.400.

2.7 Cytoscape

Clustering of PPIs was computed using the MCODE APP (version 2.0.2) in Cytoscape (version 3.9.1) (Shannon et al., 2003). Cytoscape is an open-source software for visualizing interaction networks and integrating them with various algorithms. MCODE is an app installed in Cytoscape that clusters the given network based on topology to find densely connected regions to which members might contribute as hub genes.

2.8 Animal Experiments

The Animal Care and Use Committee of the Chinese Academy of Medical Sciences and Peking Union Medical College conducted and approved all animal experiments. Male BALB/c mice weighing between 16.0 g and 19.0 g used in the first experiment were obtained from Beijing HFK Bio-Technology Co., Ltd. (Beijing, China). Male Balb/c mice weighing between 18.0 g and 21.0 g for the third experiment were obtained from the National Institutes for Food and Drug Control (Beijing, China).

For the first animal experiment, also referred to as “D14” or “late stage” (ethical inspection No.00003256). The subcutaneous tumor model was developed by implanting 0.2 mL suspensions of CT26 tissue cells into the left flank of mice, and 8 nontumor-bearing mice were used as the normal control group. After 24 h of inoculation, mice with tumors were randomly divided into 6 groups with 9 animals in each group: double distilled water (DDW) as a solvent control in Group 1, 5-FU at 30.0 mg/kg (dissolved in normal saline) in Group 2, ZYJN at 0.75 g/kg (dissolved in DDW) in Group 3, ZYJN at 1.5 g/kg (dissolved in DDW) in Group 4, ZYJN at 0.75 g/kg combined with 5-FU (30.0 mg/kg) in Group 5, and ZYJN at 1.5 g/kg combined with 5-FU (30.0 mg/kg) in Group 6. The day of implantation was recorded as day zero (D0), and all animals (except 5-FU) received administration from D0 as follows: DDW and ZYJN were administered for 14 days (qd, i.g.); 5-FU was administered...
from D1 four times (q.3d, tail vein injection). Non-anticoagulated peripheral blood was collected before euthanasia. Tumor mass, spleen and thymus were excised, weighed after euthanasia. The inhibition rate was measured as previously described (Tang et al., 2023). Parts of the tumor tissues were collected for flow cytometry, and the rest were kept at -80°C for further analysis.

For the second animal experiment, also referred to as “D11” or “early stage” (ethical inspection No.00003264). The implantation was the same as described previously. Ten nontumor-bearing mice were used as the normal control group. In addition, tumor-bearing mice were randomly divided into 4 groups with 12 mice in each group: DDW in Group 1, 50.0 mg/kg 5-FU (normal saline) in Group 2, 0.75 g/kg ZYJN (DDW) in Group 3, and 1.50 g/kg ZYJN (DDW) in Group 4. Groups 1, 3, and 4 were administered for 9 days (qd, i.g.) from D0; 5-FU was administered from D1 3 times (q.3d, tail vein injection). Sampling was performed as described previously.

For the third animal experiment (ethical inspection No.00009342). The implantation and normal control groups were the same as described before. In addition, tumor-bearing mice were randomly divided into 4 groups with 10 mice in each group: 25% PEG400 (DDW, V/V) in Group 1, 30.0 mg/kg 5-FU (normal saline) in Group 2, 0.75 g/kg ZYJN (25% PEG400) in Group 3, and 1.50 g/kg ZYJN (25% PEG400) in Group 4. Groups 1, 3, and 4 were administered for 11 days (qd, i.g.) from D1; 5-FU was administered from D1 4 times (q.3d, tail vein injection). Sampling was performed as described previously.

2.9 Western Blot

CT26 tumor tissue from the solvent control group (n=3) and ZYJN 0.75 g/kg group (n=3) were lysed with radioimmunoprecipitation assay buffer (RIPA buffer [high], Solarbio, R0010, China). Total protein concentrations were quantified using a BCA Protein Assay Kit (Solarbio, PC0020, China) to ensure equal sample loading. Proteins were separated in 8% or 10% sodium dodecyl sulfate–polyacrylamide gels and transferred onto 0.45 μm polyvinylidene difluoride (PVDF) membranes (Millipore, IPVH00010, Ireland). Next, the membrane was blocked with 5% skim milk (W/V, 1 × TBST [0.2% Tween-20 in Tris-buffered saline]) for 1 h at room temperature (R.T.) and then incubated with primary antibodies overnight at 4°C. The membranes were washed with 1 × TBST 3 × 6 minutes before incubating with peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (ZSGB-BIO, ZB-2301, ZB-2305, 1:5000, China) for 1 h at R.T. The membranes were washed with 1 × TBST 3 × 6 min before visualization using Super ECL Plus hypersensitive chemiluminescence solution (Applygen Technologies Inc., P1050, China) and detected using an Invitrogen iBright FL1000 Gel Imaging System (Thermo Fisher Scientific, USA).

Antibodies against β-actin (CST-8457, 1:1000), CCNB1 (CST-4138, 1:1000), CDK1 (CST-9116, 1:1000), p-Akt (Thr308, CST-2965, 1:1000), Akt (CST-4691, 1:1000), and PI3K-p110α (CST-4249, 1:1000) were purchased from Cell Signaling Technology (USA). Anti-MMP-9 antibody (ab38898, 1:1000) was purchased from Abcam (UK).

2.10 Flow cytometry

Freshly excised CT26 tumor tissues were dissected into small fragments and digested with type IV collagenase (0.5%) (Gibco, USA) and DNase I (0.05%) (Sigma, USA).
dissolved in RPMI-1640 (Gibco, USA) at 37°C to obtain a single-cell suspension. The cells were collected and washed, and red blood cells were lysed with ACK buffer. Cells were counted and adjusted to $1 \times 10^7$ cells per mL with 1 × PBS and divided into Eppendorf tubes at a volume of 100 μL (1 × 10^6 cells/tube). Cells were stained with Red Fixable LIVE/DEAD probe (Thermo Fisher, L34971, USA) and following surface antibodies: PerCP-Cy5.5 anti-mouse CD45 (Thermo Fisher, 45-0451-82, USA), PerCP-eFluor710 anti-mouse CD3 (Thermo Fisher, 46-0032-82, USA), PE-Cy7 anti-mouse CD4 (Thermo Fisher, 25-0042-82, USA), FITC anti-mouse CD8 (Thermo Fisher, 11-0081-82, USA), PE anti-mouse NKP46 (Thermo Fisher, 12-3351-82, USA), PerCP-eFluor710 anti-mouse CD11b (Thermo Fisher, 46-0112-82, USA), PE-Cy5 anti-mouse F4/80 (BioLegend, 123112, USA), PE anti-mouse CD206 (Thermo Fisher, 12-2061-82, USA), PE-Cy7 anti-mouse MHC II (BioLegend, 107629, USA), FITC anti-mouse Ly6G (BioLegend, 127606, USA), PE-Dazzle594 anti-mouse Ly6C (BioLegend, 128044, USA) on ice for 0.5 h in the dark. After washing with 1 × PBS (600 × g, 5 min), the cells were resuspended in 200 μL of 1 × PBS. Data acquisition and analysis were performed using a Cytek Aurora Spectral Flow Cytometer (Cytek Biosciences, USA) with FlowJo software.

2.11 Blood biochemistry

After clotting of non-anticoagulated peripheral blood for two hours at room temperature, serum was collected after centrifugation at 2000 × g for 20 min and analyzed using a Fully Automatic Biochemistry Analyzer (TOSHIBA, TBA-40FR, Japan). The Total Protein (TP) Assay Kit (Biuret method, 100000260), Albumin (ALB) determination kit (Bromocresol green method, 100020150), and Aspartic acid Aminotransferase (AST) Assay Kit (100020010) were purchased from BioSino Bio-Technology & Science Inc., China.

2.12 Luminex assay

Serum samples were collected and analyzed using the Mouse Premixed Multi-Analyte Kit (Cat: LXSAMSM-12, Lot: L140507; Cat: LXSAMSM-27, Lot: L140508, R&D Systems, USA) according to the manufacturer’s instructions. Briefly, serum samples were diluted (1:2) and incubated with antibody-coated microparticles in the dark for 2 h at room temperature on a horizontal orbital microplate shaker at 800 rpm. Each well was washed with wash buffer for 3 × 1 min on a shaker and incubated with a biotinylated antibody cocktail in the dark for 1 h at room temperature. Wells were washed again with wash buffer for 3 × 1 min before incubation with phycoerythrin (PE)-conjugated streptavidin for 30 min at room temperature on a shaker at 800 rpm. After washing with wash buffer for 3 × 1 min, microparticles in each well were resuspended in wash buffer for 2 min on the shaker at 800 rpm before the plate was run on the Luminex 200 system (Luminex Corporation, USA). The provided standard was diluted 3-fold to generate a standard curve.

2.13 Detection of peripheral blood

20 μL of mouse peripheral blood was collected and mixed thoroughly with 2 mL of blood dilution buffer and analyzed with an automatic hematology analyzer (NIHON KOHDEN, Mek-680, Japan).

2.14 Transparent imaging analysis

The mice were anesthetized, and then 0.2 mL of 637 blood vessel marker (Thousand
Dimensions, td210007, China) was injected into the tail vein of each mouse for 3 min.

After myocardial perfusion, the tumor tissues were collected and fixed. The tumor tissues were rinsed with 20 mL of 0.01 mol/L PBS for 3 × 20 min and then gradually defatted with 30%, 50%, and 70% tert-butanol solution. The tissues were dehydrated and matched to a uniform refractive index at 37°C on a shaker at 40 rpm to achieve tissue transparency. A Nuohai LS18 transparent optical microimaging system (Nuohai Life Science, Co., Ltd, China) was used to image the tumor tissues at a resolution of 1.65 μm × 1.65 μm × 3.5 μm, which could clearly distinguish the blood vessels and fibers in the whole tumor tissue. The data processing included preprocessing of the original image data, stitching and combining of thumbnail images, brightness correction, and other corrections such as image background fluorescence elimination and image rotation correction to optimize the two-dimensional images. After three-dimensional data reconstruction, the relevant parameters of the blood vessels and fibers in the tumor tissues were calculated, and quantitative analysis data were collected using Imaris 3D analysis software (Bitplane, UK). Statistical charts were generated from the quantitative data using TD ANA 1.0 software (Thousand Dimensions, China).

2.15 Statistical analysis

Data are presented as the mean ± standard deviation, and the statistical analysis was conducted using Microsoft Office Excel 2019 (Microsoft Corp., USA) and SPSS statistical software (Version 20.0, SPSS Inc, USA). Student's two-tailed t test was used to make comparisons between two groups, and one-way analysis of variance was performed to evaluate the differences between multiple groups. P < 0.05 was considered statistically significant.

3. Results

3.1 Fingerprint analysis of ZYJN

HPLC was utilized to analyze the chemical constituents of ZYJN (Figure 1). By comparing the retention time of chromatographic peaks of the reference substance, 12 chromatographic peaks were identified with the B7 chromatographic peak with a stable peak and large peak area as the reference peak (Figure 1A). Calycosin-7-O-β-D-glucoside (peak B1, CAS number: 20633-67-4), nuezhenide (peak B2, CAS number: 39011-92-2), hesperidin (peak B3, CAS number: 520-26-3), epimedin A (peak B4, CAS number: 110623-72-8), epimedin B (peak B5, CAS number: 110623-74-9), epimedin C (peak B6, CAS number: 110642-44-9), icariin (peak B7, CAS number: 489-32-7), nobiletin (peak B8, CAS number: 478-01-3), baohuoside I (peak B9, CAS number: 113558-15-9), atractylenolide III (B10 peak, CAS number: 73030-71-4), tangeretin (B11 peak, CAS number: 481-53-8), oleanolic acid (B12 peak, CAS number: 508-02-1). ZYJN from 10 batches was analysed by HPLC to validate the reliability and repeatability of the established method (Figure. 1B).

According to TCMSP, B1 might come from Astragalus mongholicus Bunge. B2 and B12 may be derived from Ligustrum lucidum Ait. B3, B8 and B11 may come from Citrus reticulata Blanco. B4, B5, B6, B7, B9 and B12 may be derived from Epimedium sagittatum (Sieb. et Zucc.) Maxim. B10 may be derived from Atractylodes macrocephala Koidz.
Figure 1. Fingerprint analysis of ZYJN. (A) Chemical constituents in ZYJN identified by HPLC at 203 nm. (B) Fingerprint of ZYJN from 10 batches analyzed by HPLC. S1: 51191234; S2: 51191235; S3: 51191236; S4: 51191237; S5: 51191239; S6: 51191241; S7: 51191244; S8: 51200102; S9: 51200105; S10: 51200107.

3.2 The predictive targets of ZYJN components for treating colorectal cancer

By predicting possible targets of these 12 components on the Swiss Target Prediction website, a total of 221 potential targets were identified. The PPI network was established on the STRING website (Figure 2), and then MCODE installed in Cytoscape was applied to calculate the hub gene, and the gene in the cluster with the highest score was selected as the hub gene. A total of 25 hub genes were obtained (Figure 3).
Figure 2. PPI network analysis of 221 potential targets of ZYJN.

Figure 3. Top 25 hub genes calculated by Cytoscape.
3.3 ZYJN inhibited the tumor growth of CT26 colorectal cancer in the early stage

To determine the antitumor activity of ZYJN in vivo, subcutaneously transplanted mouse CT26 models were constructed; nontumor-bearing mice were treated as the normal control, and mice administered with DDW were treated as the solvent control. After administration of ZYJN for 14 days, the combination group of ZYJN 0.75 g/kg exhibited a better tumor growth-inhibitory effect (with an inhibition rate of 81.13%) compared with the combination group of ZYJN 1.50 g/kg (with an inhibition rate of 78.15%) and the 5-FU single group (with an inhibition rate of 70.24%) (Figure 4A). The single use of ZYJN did not show a significant inhibitory effect on tumor growth on D14. However, we did observe the inhibition effects of the ZYJN single group calculated by tumor volume on D10 (Figure 4D). Therefore, we performed another experiment to validate the antitumor effects of ZYJN in a mouse CT26 model. After administration of ZYJN for 11 days, ZYJN inhibited the tumor growth of CT26 cells in a dose-dependent manner. The tumor weight inhibitory rate of ZYJN 1.50 g/kg was 41.68%, while the tumor weight inhibitory rate of ZYJN 0.75 g/kg was 21.95%. The inhibition rate of 5-FU 50.0 mg/kg was 85.23% (Fig. 4B). However, in the last experiment, we found that the inhibitory effect of ZYJN on the tumor weight remained basically unchanged at 0.75 g/kg (22.9%), while ZYJN 1.50 g/kg showed no tumor weight inhibitory effect (1.2%). The inhibition rate of 5-FU 30.0 mg/kg was 82.9% (Figure 4C).

Previous studies have demonstrated that tissue inhibitor of metalloproteinases-1 (TIMP-1) is a biomarker in several types of cancer. In our study, we observed a significant increase in TIMP-1 serum concentration in the solvent control group compared with the normal control group. ZYJN showed a tendency to decrease TIMP-1 (with no statistical significance) in the early stage of the CT26 model (D11, Figure 5B), while this tendency was less obvious in the late stage (D14, Figure 5A). With regard to the 5-FU groups, the single and combination groups of 5-FU showed a significant decrease in the serum concentration of TIMP-1 (Figure 5B). The protumorigenic properties of chemokine (C-C motif) ligand 7 (CCL7) have been confirmed in CRC cells. We observed a significant increase in CCL7 serum concentration in the solvent control group compared with the normal control group (Figure 6B). The administration of ZYJN decreased the serum concentration of CCL7 in the early stage of the CT26 model (D11, Figure 6B) and this effects were
less obvious in the late stage of the CT26 model (D14, Figure 6A).

Figure 5. Serum concentration of TIMP-1 in the first animal experiment (A) and the second (B).
▲▲▲ \( P < 0.001 \) versus Normal, \( * P < 0.05, ** P < 0.05, *** P < 0.001 \) versus Solvent control.

Figure 6. Serum concentration of CCL7/MCP-3/MARC in the first animal experiment (A) and the second (B).
▲▲▲ \( P < 0.001 \) versus Normal.

3.4 ZYJN had protective effects on tumor-bearing mice

In the combination experiments, compared with the normal control group, the solvent control group exhibited a decrease in the serum concentration of total protein (TP) (Figure 7A) and ALB (Figure 7B). Interestingly, the combination of ZYJN and 5-FU could increase the serum level of ALB, while the single use of ZYJN showed no significant difference in D14; the solvent control group showed a significant increase in AST (Figure 7C) compared with the normal control group; the combination therapy had a better effect in lowering AST than the monotherapy of 5-FU. The administration of ZYJN alone showed no influence on the body weight of tumor-bearing mice, while the other three groups treated with 5-FU 30.0 mg/kg showed a slower increase pattern in body weight than ZYJN alone (Figure 8A). In the single administration experiment, mice treated with 5-FU at 50.0 mg/kg showed a significant decrease in the body weight of tumor-bearing mice after D4; mice treated with ZYJN showed a steady increase pattern in body weight, similar to the solvent and normal
control groups (Figure 8B).

Figure 7. Serum levels of TP (A), AST (B) and ALB (C) in normal mice and tumor-bearing mice.

▲▲ P < 0.01, ▲▲▲ P < 0.001 versus Normal, *P < 0.05 versus Solvent control.

Figure 8. Changes in body weight of mice during the animal experiment procedure. first animal experiment (A) and second (B).

* P < 0.05, ** P < 0.01, *** P < 0.001 versus Solvent control.

Peripheral blood was analyzed to understand the effects of ZYJN and 5-FU. The white blood cell count (WBC) of tumor-bearing mice was not significantly different from that of normal mice but was decreased in mice treated with 5-FU, while the WBC of mice was increased in the ZYJN 1.50 g/kg group (Figure. 9A). The red blood cell count (RBC) of tumor-bearing mice was significantly lower than that of normal mice, while no differences were observed in tumor-bearing mice (Figure. 9B). ZYJN had a profound effect on platelets (PLT). The PLT of tumor-bearing mice was lower than that of normal mice, and the PLT of 5-FU-treated mice was significantly improved. However, the combination of ZYJN was significantly higher than 5-FU and tumor-bearing mice (Figure. 9C).
Figure 9. Concentration of WBCs (A), RBCs (B) and PLTs (C) in peripheral blood. **P < 0.01 versus Solvent control, ▲P < 0.01 versus Normal.

3.5 ZYJN could inhibit angiogenesis in tumors

To examine whether ZYJN could inhibit the angiogenesis of colorectal cancer, tissue clearing and fluorescence microscopic tissue imaging were used to identify the angiogenesis of cancer tissues. As shown in Figure 10, administration of 5-FU showed no significant difference compared with the solvent control group. The area under the curve (AUC) of the combination group of ZYJN 1.50 g/kg was higher than that of the solvent control group and 5-FU group, which indicated that the integrity of blood vessels within tumor tissues was disrupted by the combination of ZYJN and 5-FU. The Luminex assay demonstrated that the solvent control group showed a significant increase in VEGF concentration compared with the normal control group, while the administration of 5-FU decreased the serum concentration of VEGF compared with the solvent control group. ZYJN 1.50 g/kg also decreased the serum concentration of VEGF. The two combination groups had decreased VEGF concentrations, but they showed no significant difference when compared with the 5-FU group (Figure. 11). Angiopoietin-2 expression is usually upregulated in a wide range of human cancers and is related to tumor angiogenesis, so we examined the concentration of angiopoietin-2 in serum. The combination of ZYJN 1.50 g/kg and 5-FU significantly decreased the serum concentration of angiopoietin-2 in the late stage, which was consistent with the result that this group exhibited the best tumor inhibition effect. However, mice treated with 5-FU or ZYJN showed no difference in serum angiopoietin-2 levels compared to the solvent control group in the early stage of CT26 model (Figure. 11).
Figure 10. Top: In this statistical chart of tumor vascular tissue length, the significant differences among each group and each stage were analyzed, represented by the $P$ value. That is, $^*P < 0.05$, $^{***}P < 0.001$, 5-FU group versus Solvent control; $\Delta P < 0.05$, $\Delta\Delta\Delta P < 0.001$, ZYJN 0.75 g/kg group versus Solvent control; $^*P < 0.05$, $^{***}P < 0.001$, 5-FU + ZYJN 1.50 g/kg group versus Solvent control. Bottom: The picture in this figure shows the data of tumor vascular tissue after hyalinization and imaging collection in each group. Al, B1, C1, D1, and El are physiological state images after hyalinization. The resolutions adopted were as follows: A2-A4, B2-B4, C2-C4, D2-D4, and E2-E4. (1.65*1.65*3.5) μm, NUOHAI LIFE SCIENCE LS 18 transparent light microscopy imaging system after tumor tissue imaging two-dimensional global projection and three-dimensional recomposition, can clearly distinguish the entire tumor tissue vasculature. A5, B5, C5, D5, and E5 are fiber reconstructions for tumor vascular tracking using Imairs data processing software.
3.6 ZYJN enhanced the fibrosis process to inhibit tumor invasion

In 3D imaging experiments, the AUC of the diameter and length of fiber tissues is an effective way to evaluate the number of fiber tissues around the tumor tissues. The AUC of groups treated with ZYJN (0.75 g/kg) or groups treated with the combination of 5-FU and ZYJN (1.50 g/kg) was greater than that of groups treated with 5-FU alone, which demonstrates that ZYJN can boost the fibrosis process around the tumor tissues and thus exert an inhibitory effect on tumor invasion (Figure 12).

Figure 12. Serum concentration of VEGF and angiopoietin-2 in CT26 model. (A) Serum concentration of VEGF in the first experiment. (B) Serum concentration of angiopoietin-2 in the first experiment. (C) Serum concentration of VEGF in the second experiment. (D) Serum concentration of angiopoietin-2 in the second experiment. *P < 0.05, **P < 0.01, ***P < 0.001 versus Solvent control. ▲P < 0.05 versus Normal.

**Figure 11.** Serum concentration of VEGF and angiopoietin-2 in CT26 model. (A) Serum concentration of VEGF in the first experiment. (B) Serum concentration of angiopoietin-2 in the first experiment. (C) Serum concentration of VEGF in the second experiment. (D) Serum concentration of angiopoietin-2 in the second experiment. *P < 0.05, **P < 0.01, ***P < 0.001 versus Solvent control.

**Figure 12.** Top: In this statistical chart of tumor fiber tissue diameter (left) and tumor fiber tissue length (right), the significant differences among each group and each stage were analyzed by P value. That is, **P < 0.01, ***P < 0.001, versus 5-FU group; *P < 0.05, **P < 0.01, ***P < 0.001, versus ZYJN 0.75 g/kg group; ▲P < 0.05, ▲▲▲P < 0.001, versus ZYJN 1.50 g/kg group; ★P < 0.05, ★★P < 0.01, ★★★P < 0.001, 5-FU + ZYJN 1.50 g/kg group versus Solvent control group. Bottom: The picture
478 in this figure shows the data of tumor fiber tissue after hyalinization and imaging collection in
479 each group. Al, B1, C1, D1, and E1 are physiological state images after hyalinization. The
480 resolutions adopted were as follows: A2-A4, B2-B4, C2-C4, D2-D4, and E2-E4. (1.65*1.65*3.5)
481 μm, NUOHAI LIFE SCIENCE LS 18 transparent light microscopy imaging system after tumor
482 tissue imaging two-dimensional global projection and three-dimensional recomposition, can
483 clearly distinguish the entire tumor tissue fibers. A5, B5, C5, D5, and E5 are fiber reconstructions
484 for tumor fiber tracking using Imairs data processing software.
485
486 3.7 ZYJN may affect multiple protein pathways
487
488 Based on bioinformatic analysis of potential targets of ZYJN, changes in the
489 expression of proteins related to the cell cycle, cell proliferation and cell migration
490 were detected.
491
492 Reduced expression of PI3K-p110α was observed in the ZYJN 0.75 g/kg group.
493 Although downstream Akt was almost unaffected by ZYJN, the phosphorylated active
494 form, p-Akt (Thr308), was inhibited, indicating a decreased proportion of active Akt.
495 On the other hand, the expression of CDK1 and CCNB1 was significantly decreased
496 in the ZYJN 0.75 g/kg group, and the expression of MMP-9 was also significantly
497 decreased (Figure 13).
498
499 Figure 13. Western blot analysis of PI3K-p110α, p-Akt (Thr308) and Akt (left); CCNB1 and
500 CDK1 (middle); MMP-9 (right).
501
502 3.8 ZYJN had positive effects on the immune constitution of the tumor
503 microenvironment
504
505 The tumor microenvironment (TME) refers to the complex and multicellular
506 environment in which a tumor develops. Here, flow cytometry was used to investigate
507 the immune constituents of the TME within CT26 colorectal cancer. The percentage
508 of tumor-infiltrating T cells and CD4+ T cells in the two combination groups was
509 significantly higher than that in the solvent control group and 5-FU group in the late
510 stage of the mouse CT26 Model (D14) of cancer (Figure. 14B and 14H). Interestingly,
511 the percentages of tumor-infiltrating CD4+ T cells in the 5-FU group were higher than
512 those in the solvent control group in the early stage of the mouse CT26 model of
513 cancer (D11, Figure. 14C). Previous studies have demonstrated the critical role of
514 host cytotoxic T lymphocyte and NK cell-mediated effector functions in cancer
515 resistance. Mice treated with 0.75 g/kg or 1.50 g/kg ZYJN showed an increase in the
516 percentage of cytotoxic T lymphocytes (CD45+CD3+CD8+) in the early stage of the
517 mouse CT26 model of cancer (Figure. 14G). In the early stage, the administration of
518 ZYJN (1.50 g/kg) showed a significant increase in the percentage of tumor-infiltrating
519 NK cells compared with the solvent control group (Figure. 14E). Our results were
520 consistent with previous studies that demonstrated that NK and T cells in the TME
521 were more likely to change in the early stage of mouse cancer models, while tumor
522 infiltrating myeloid-derived suppressor cells (MDSCs) were more likely to change in
the late stage. As important immunosuppressive cells in the TME, MDSCs were examined. We observed an increase in the percentage of M-MDSCs in groups treated with ZYJN or 5-FU in the early stage (D11, Figure. 15C) and late (D14, Figure. 15B) stage of the mouse CT26 model of cancer compared with the solvent control group. Groups treated with ZYJN or 5-FU showed a significant decrease in the percentage of PMN-MDSCs in the late stage of the mouse CT26 model of cancer (Figure. 15D). The combination of ZYJN (1.50 g/kg) and 5-FU showed better effects in decreasing the percentage of total MDSCs (Figure. 15J), which demonstrated that the combination of ZYJN and 5-FU helped to relieve the immunosuppressive TME in the mouse CT26 model. Moreover, we observed interesting changes in the percentage of M1 and M2 macrophages. An increasing number of studies have indicated that TAMs found in the TME are mostly M2 macrophages, and the presence of abundant M2 macrophages in the TME resulted in the initiation and continuous growth of tumors, while we observed that the M1 type was much more abundant than the M2 type in the early stage of the mouse CT26 model of cancer (Figure.15G and 15I). Interestingly, there were more M2 macrophages than M1 macrophages in the solvent control groups in the late stage (Figure. 15F and 15H). Mice treated with 5-FU or ZYJN showed an increase in the percentage of M1 macrophages compared with the solvent control group, especially in the late stage (Figure. 15F).

Figure 14. (A) Gating strategy of T cells and NK cells. (E) Gating strategy of macrophages and MDSCs. (B and C) The percentages of CD4+ T cells; (D and E) The percentage of CD8+ T cells; (F and G) The percentage of NK cells; (H and I) The percentage of T cells, CD45 gated. *P < 0.05, **P < 0.01, ***P < 0.001 versus Solvent control.
Figure 15. (A) Gating strategy of macrophages and MDSCs. (B and C) The percentages of MMDSCs; (D and E) The percentage of PMN-MDSCs; (F and G) The percentage of M1 macrophages; (H and I) The percentage of M2 macrophages; (J and K) The percentage of total MDSCs, CD45 gated. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus Solvent control, #*$P < 0.05$, ##*$P < 0.01$ versus 5-FU.

4. Discussion

In this article, we performed three batches of experiments to explore the efficacy and mechanism of monotherapy and combination therapy with ZYJN.

We believe that fibrosis of the tumor promotes its integrity and reduces metastasis. The 0.75 g/kg dose of ZYJN alone increased the fibrous tissue in the tumor, which may be related to the decrease in MMPs. Although ZYJN alone did not inhibit MMP-12 expression significantly, it overcame individual differences in the high expression of MMP-12 in tumor-bearing mice (S1.). ZYJN also inhibited the expression of MMP-9 protein in vivo.

As a natural inhibitor of MMP, TIMP-1 was once thought to be a marker for inhibiting cancer invasion and metastasis. However, recent studies have shown that high expression of TIMP-1 may also be positively correlated with poor prognosis in some cancers (Eckfeld et al., 2019). The Luminex assay showed that the serum concentration of TIMP-1 in tumor-bearing mice was higher than that in normal mice. Similarly, ZYJN alone can reduce TIMP-1 while inhibiting tumor growth. On the other hand, studies revealed that the microvascular content of TIMP-1-deficient mice was reduced and vascular remodeling was impaired, suggesting that TIMP-1 may be...
involved in promoting maturation of the vascular network (Mandel et al., 2017). In
the vascular hyalinization experiment, the AUC of ZYJN monotherapy was smaller
than that of the solvent control group and 5-FU group, and the number of blood
vessels in the tumor might be reduced. As VEGF is typically a pro-angiogenic factor
(Apte et al., 2019), an increase in angiopoietin-2 has also been observed in tumors
(Nicolini et al., 2019), and the different changes in the blood vessels in the tumor may
be related to the different levels of TIMP-1, VEGF and angiopoietin-2.

ICAM/CD54 is an important molecule that mediates cell adhesion and can be
expressed in a variety of malignant tumors and immune cells, mediating
hematogenous metastasis of cancer cells and recruitment of immune cells (Bui et al.,
2020). YAP/TAZ knockout impaired the antitumor capacity of CD54+ neutrophils,
and the relative number of cells was negatively correlated with gastric cancer
progression (Nie et al., 2022). We found that ZYJN can reduce the expression of
ICAM-1 (S2.), suggesting that ZYJN may affect the migration and invasion of CRC
cells, which is consistent with the above results, and may also affect the tumor
immune microenvironment.

CCL7 secreted by mesenchymal stem cells (Xu et al., 2022) and M-MDSCs (Ren et
al., 2021) in the tumor microenvironment can promote CRC progression and
metastasis and may also be involved in osteolysis after CRC bone metastasis (Yang et
al., 2022). Although ZYJN did not affect serum CCL7 levels in the late stage, its early
inhibitory effect may still affect CRC progression.

IL-10 is a dual immune-functional factor. On the one hand, it can inhibit the
production of IFN-γ by activating human PBMCs and inactivating T cells. On the
other hand, it can also act in conjunction with other factors to stimulate the survival
and activation of immune cells (Mannino et al., 2015). We found that IL-10
expression was increased in CRC compared to normal mice (S3), and IL-10 was
decreased to varying degrees after administration of ZYJN alone, suggesting that IL-
10 may play an immunosuppressive role in CRC and that ZYNJ may alleviate this,
which can be demonstrated in FCM as discussed later in this section.

In peripheral blood cell analysis, ZYJN significantly increased WBCs at a 1.50 g/kg
dose, suggesting a possible immune-boosting effect. Similarly, a high dose of ZYJN
monotherapy significantly increased the percentage of tumor-infiltrating CD8+ T cells
and NK cells, while a 0.75 g/kg dose also had some effect on these two cell types,
suggesting that ZYJN may inhibit tumor growth by increasing the proportion of
antitumor immune cells.

In addition, Western blot assays showed that the PI3K-Akt signaling pathway was
inhibited, and the expression of CDK1 and CCNB1 at the G2/M checkpoint was
decreased in tumors administered ZYJN. These results suggested that ZYJN could
block tumor cells at the G2/M stage by inhibiting the PI3K-Akt pathway and
ultimately inhibit tumor growth and proliferation.

Although the body weight of the combination group was slightly lower than that of
the 5-FU group, the body weight of these mice showed an overall upward trend
during the experimental period. The combination group of ZYJN 0.75 g/kg and 1.50
g/kg showed similar tumor inhibition rates. ZYJN 1.50 g/kg in combination with 5-
FU increased the fibrous tissue in the tumor. Similarly, monotherapy and combination
therapy with ZYJN could reduce the serum concentration of TIMP-1 and inhibit
tumor growth. The ZYJN combined with 5-FU group showed obvious bimodality,
indicating that the combination of ZYJN combined with 5-FU destroyed the integrity of blood vessels in tumor tissue. IL-10 was decreased to varying degrees after administration of ZYJN in combination with 5-FU (S3.). Above all, ZYJN combined with chemotherapy had a certain synergistic effect.

ZYJN in combination with 5-FU also had attenuated effects. AST associated with liver and heart function impairment was significantly increased in tumor-bearing mice compared with normal mice, while the combination of 1.50 g/kg ZYJN with 5-FU significantly decreased AST content, which was significantly lower than that of 5-FU.

ALB was significantly decreased in the serum of tumor-bearing mice, suggesting the possibility of cachexia, and significantly increased after combination therapy, reducing the possibility of cachexia in mice.

Unfortunately, the ZYJN combination had adverse effects on platelets, and the PLT increased significantly after the combination and exceeded that of normal mice, which may increase the risk of excessive platelets. The reason for the upregulation of platelet content by the combination needs to be further investigated.

5. Conclusions

In conclusion, ZYJN, as a Chinese traditional medicine compound, has certain early anti-colorectal cancer effects by promoting tumor fibrosis, suppressing angiogenesis, migration, and invasion and modulating the tumor immune microenvironment. ZYJN enhanced the efficacy and reduced the toxicity of chemotherapy drugs in combination therapy, and the mechanism remains to be further explored. Our studies highlighted that ZYJN is a potential drug for cancer treatment.

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CRediT authorship contribution statement

Haidong Deng: Formal analysis, Investigation, Writing - original draft; Siqi Liu: Formal analysis, Data curation, Writing - original draft; Didi Li: Formal analysis, Writing - original draft; Weiping Wang: Investigation; Ling Ye: Writing – review & editing; Shaofeng Xu: Investigation; Xiaoliang Wang: Project administration, Funding acquisition; Yan Li: Validation, Funding acquisition.

Declaration of competing interest

The authors declared that there is no conflict of interest.

Abbreviations

<table>
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<tr>
<th>Abbreviation</th>
<th>Full names</th>
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<tr>
<td>5-FU</td>
<td>5-Fluorouracil</td>
</tr>
<tr>
<td>ALB</td>
<td>Albumin</td>
</tr>
<tr>
<td>AST</td>
<td>Aspartic acid Aminotransferase</td>
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<td>AUC</td>
<td>Area under the curve</td>
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Supplementary
References


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Shannon, P., Markiel, A., Ozier, O., Baliga, N.S., Wang, J.T., Ramage, D., Amin, N.,


Szklarczyk, D., Gable, A.L., Lyon, D., Junge, A., Wyder, S., Huerta-Cepas, J., Simonovic, M.,
protein association networks with increased coverage, supporting functional discovery in

suppressed glioma tumor growth by downregulating the EGFR/PI3K/AKT/mTOR signaling
pathway and modulating the tumor microenvironment. Toxicol Appl Pharmacol 460, 116378.

Xu, Z., Gao, H., Zhang, Y., Feng, W., Miao, Y., Xu, Z., Li, W., Chen, F., Lv, Z., Huo, J., Liu, W.,
Shen, X., Zong, Y., Zhao, J., Lu, A., 2022. CCL7 and TGF-β secreted by MSCs play opposite
roles in regulating CRC metastasis in a KLF5/CXCL5-dependent manner. Mol Ther 30(6),
2327-2341.

Yang, H., Jian, L., Jin, Q., Xia, K., Cai-Ru, W., Jun, S., Chen, H., Wei, W., Ben-Jing, S., Shi-
Hong, L., Shi-Wei, L., Juan, W., Wei, Z., 2022. CCL7 playing a dominant role in recruiting early
OCPs to facilitate osteolysis at metastatic site of colorectal cancer. Cell Commun Signal 20(1),
94.