European Journal of Pharmacology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Full length article

An apple oligogalactan enhances the growth inhibitory effect of 5-fluorouracil on colorectal cancer

Yuhua Li[a,c,1], Lei Fan[d,1], Yinbo Niu[e], Wenguang Mian[c], Feng Zhang[b], Ming Xie[c], Yang Sun[b,*], Qibing Mei[a,b,**]

[a] Laboratory of Oncological Pharmacology, Department of Pharmacology, School of Pharmacy, Southwest Medical University, Luzhou 646000, Sichuan, PR China
[b] Key Laboratory of Gastrointestinal Pharmacology of Chinese Materia Medica of the State Administration of Traditional Chinese Medicine, Department of Pharmacology, School of Pharmacy, The Fourth Military Medical University, Xi'an 710032, Shaanxi, PR China
[c] No.422 Hospital of PLA, Zhanjiang 524005, Guangdong, PR China
[d] Department of Pharmacy, No. 210 Hospital of PLA, Liaoning 116000, PR China
[e] School of Life Sciences, Northwestern Polytechnical University, Xi'an, Shaanxi 710072, PR China

ARTICLE INFO

Keywords:
An apple oligogalactan (AOG)
5-FU
Colorectal cancer (CRC)

ABSTRACT

Treatment of colorectal cancer (CRC) remains a clinical challenge, since current therapies are associated with obvious side effects and high expenses. These limitations highlight an urgent need for developing novel and safe treatment strategies. It is suggested that combinatorial strategies could be more effective and much safer than monotherapy in cancer treatment. In our previous study, an apple oligogalactan (AOG) has been found to show beneficial effect on treating CRC. This study tried to investigate whether AOG could enhance the growth inhibitory effect of 5-FU in human CRC cells (HT-29 and SW-620), a mouse model of colitis associated colorectal cancer and a murine model of xenograft tumor. The IC_{50} values of 5-FU were 26.70 ± 0.21 μM in HT-29 cells and 26.71 ± 2.06 μM in SW-620 cells. Pretreatment with 0.05 or 0.1 mM AOG down-regulated IC_{50} values of 5-FU to 22.44 ± 1.01 or 18.67 ± 1.16 μM in HT-29 and 21.21 ± 1.49 or 17.99 ± 1.42 μM in SW-620 cells. AOG enhanced 5-FU-induced cell apoptosis and S phase arrest. The combination not only protected ICR mice against intestinal toxicities and carcinogenesis induced by 1,2-dimethylhydrazine and dextran sodium sulfate, but also decreased the xenograft tumor size, triggered apoptosis and inhibited proliferation of tumor cells in nude mice. The mechanisms of AOG on enhancing the growth inhibitory effect of 5-FU may be through the influence of TLR-4/NF-κB pathway. Taken together, the combinatorial therapy using AOG and 5-FU is a promising strategy for the treatment of colorectal cancer.

1. Introduction

Colorectal cancer (CRC) continues to be a worldwide killer, despite that the enormous amount of research and rapid development witnessed during the past decade. According to recent statistics, CRC is one of the most common cancers and a leading cause of cancer-related mortality, with an estimated 1.2 million new cases and 693,900 deaths (Jemal et al., 2011). CRC mortality has been increasing in many Western countries (Center et al., 2009). In 2014, there were an estimated 136,830 new cases of CRC and 50,310 patients died from CRC in the United States (American Cancer Society, 2013). 5-Fluorouracil (5-FU) is one of the classical drugs used as chemotherapeutic agent to treat CRC. It is converted intracellularly to different active metabolites: fluorodeoxyuridine monophosphate, fluorodeoxyuridine triphosphate and fluorouridine triphosphate—these active metabolites disrupt RNA synthesis and the action of thymidylate synthase. However, the long-term use of 5-FU is limited, mainly due to its adverse effects, including myelotoxicity and GI toxicities (e.g., diarrhea and stomatitis) (Lokich et al., 1989; Meta-analysis Group In Cancer, 1998). There is an imperative and urgent need for identifying and developing novel and safe treatment strategies.

The role of environment and lifestyle in the genesis and progression of CRC has drawn increasing clinical and scientific attention (Hogg, 2007; Divisi et al., 2006). Epidemiological studies indicate that regular

* Corresponding author.
** Corresponding author at: Laboratory of Oncological Pharmacology, Department of Pharmacology, School of Pharmacy, Southwest Medical University, Luzhou 646000, Sichuan, PR China.
E-mail addresses: ysun1145@163.com (Y. Sun), qbmei53@hotmail.com (Q. Mei).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.ejphar.2017.04.001
Received 4 November 2016; Received in revised form 30 March 2017; Accepted 3 April 2017

consumption of apples may reduce the risk of CRC (Jedrychowski and Maugeri, 2009; Jedrychowski et al., 2010; Gerhauser, 2008). An in vitro study indicates that apple extracts have the capability of suppressing tumor necrosis factor (TNF)-α induced nuclear factor (NF)-κB activation in MCF-7 cells by restraining the proteasomal activities (Yoon and Liu, 2007). A growing body of evidence has suggested many ingredients from apple have antitumor effect as well; apple flavanoids and polyphenols mainly provide the benefits of antioxidant and anticancer (He and Liu, 2008). Cloudy apple juice is observed to decrease DNA damage, hyperproliferation, and aberrant crypt foci development in the distal colon of dimethylhydrazine (DMH)-initiated rats (Barth et al., 2005). One component that makes apple juice cloudy is saccharides. In our previous study, we have found that an apple oligogalactan (AOG) effectively suppresses endotoxin-induced cyclooxygenase-2 expression, and potentiates the growth inhibitory effect of celecoxib on CRC (Liu et al., 2010).

Both the mechanism of carcinogenesis and the effect of saccharides, however, are complicated and remain to be further explored. Herein, the aim of the present study was to investigate the strategy of using AOG as a synergistic agent of 5-FU and elucidate their modes of action. And we found that AOG in combination with 5-FU significantly suppressed proliferation, induced apoptosis and cell cycle arrest in CRC cells, and possessed a growth inhibitory effect on a murine model of xenograft tumor. In addition, it showed a protective efficacy on intestinal toxicities and carcinogenesis in a colitis associated colorectal cancer (CACC) mouse model.

2. Materials and methods

2.1. Chemicals and reagents

1,2-Dimethyl-hydrazine (DMH), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 5-fluorouracil (5-FU), penicillin, and streptomycin were purchased from Sigma (St. Louis, MO, USA). Dextran sodium sulfate (DSS) with a molecular weight of 36,000–50,000 was purchased from MP Biochemicals (CA, USA). Antibodies of anti-cleaved-caspase-3, cleaved-caspase-8, cleaved-caspase-9, NF-κBp65, IκB-α, and phosphorylated form of IκB-α were purchased from Cell Signaling (Beverly, MA, USA). Antibodies of anti-CDK-2 and Cyclin A were obtained from Abcam (Cambridge, UK). Antibodies of anti-Bcl-2 and Bax were obtained from Santa Cruz Biotechnology (CA, USA). Goat anti-mouse IgG, goat anti-rabbit IgG, and fetal calf serum were purchased from Thermo Fisher Scientific (Rockford, IL, USA). Z-VAD-FMK was from Beyotime Institute of Biotechnology (Jiangsu, China). Lumigen TMA-6 kit was purchased from GE (London, UK).

2.2. Preparation of AOG

Apple polysaccharides were extracted and then AOG was obtained according to the method as previously described (Liu et al., 2010). The data in Fig. 1 indicates that molecular formula of AOG is $C_{30}H_{42}O_{31}$, with a molecular weight of 898 and a purity of > 99% evaluated by Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry analysis and ^{1}H-nuclear magnetic resonance analysis.

2.3. Cell proliferation assay

Human CRC cell lines HT-29 and SW-620 were obtained from the American Type Culture Collection (ATCC). The effect of 5-FU, AOG and their combination on viability of the cells was determined by MTT assays. Briefly, the cells were plated in 96-well plates at a density of 1×10^4 cells/well and allowed to grow for 24 h. The cells were then exposed to different concentrations of 5-FU or AOG for 24 h to determine the half maximal inhibitory concentrations (IC_{50}). In another set of experiments, cells were pretreated with 0.05 mM or 0.1 mM AOG for 4 h and then co-treated with different concentrations of 5-FU (1, 3, 10, 30, and 100 μM) for 24 h to determine optimum dose for the combinatorial treatment. 20 μl of MTT solution (5 mg/ml) was added to each well and the plate was incubated for 4 h. And the formazan was resuspended in 150 μl DMSO. Absorbance was measured at 490 nm by using a Bio-Rad ELISA reader.

2.4. Flow cytometric assays for annexin-V and nuclear DNA content distribution detection

Cell apoptosis was detected using an annexin V-FITC/PI apoptosis detection kit (BD Biosciences Pharmingen, San Diego, CA, USA) as previously described (Li et al., 2009). The fluorescent intensity was examined by flow cytometry (FACSCalibur, Becton Dickinson, San Jose, CA, USA).

2.5. Caspase-3 activity

Caspase-3 activity was detected using the Caspase-3 Activity Assay Kit (Beyotime Institute of Biotechnology, Jiangsu, China). The assay is based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) by caspase-3, leading to the release of a pNA moiety. Absorbance values were measured at 405 nm. Results were adjusted to the total protein content, and activity was expressed as nanomoles of pNA per milligram of total protein.

2.6. Co-immunoprecipitation assay

Three micrograms of primary antibody was added to 500 μg of extracted proteins. The mixture was then incubated at 4 °C for 60 min with gentle rocking. Subsequently, 20 μl of Protein A/G Plus-Agarose beads was added and incubated at 4 °C overnight. The mixture was centrifuged at 500*g* for 5 min at 4 °C. The supernatant was discarded, and the Co-IP products were washed three times with PBS. Finally, the precipitates were re-suspended in 40 μl of sample buffer for Western blot.

2.7. Induction of a mouse model of colitis associated colorectal cancer

All animal experiments were approved by the Ethical Committee for Animal Care and Use of the Fourth Military Medical University.

Fifty male ICR mice aged 5 weeks were obtained from Slaccas Experimental Animal Inc. (Shanghai, China). The mice were treated as described previously (Liu et al., 2010; Li et al., 2009). Briefly, forty mice were injected intraperitoneally with a single dose of DMH (15 mg/kg). One week later, the mice were given a course of 2% DSS in sterile non-acidified drinking water for 5 days followed by pure drinking water for 3 days in total of three courses. The forty mice were then divided into four groups equally: model, 5-FU, AOG, and 5-FU plus AOG groups. For control group (ten mice), the same procedure was performed with intraperitoneal normal saline and drinking pure water instead of DMH/DSS treatment.

2.8. Treatments for ICR mice

The mice in control and model groups were not given further treatment. The mice in the rest groups were administered through tail intravenous injection with 5-FU (20 mg/kg), AOG (20 mg/kg) or 5-FU (20 mg/kg) plus AOG (20 mg/kg) respectively every four days, from the 12th week to the end of experiment. All mice were killed in the 20th week by ether overdose. 4 cm length of colon were excised, weighed, and fixed in 10% of neutral buffered formalin for histopathological examination. The colonic mucosa was carefully scraped off with a glass slide, snap-frozen in liquid nitrogen, and stored at −80 °C until processed.

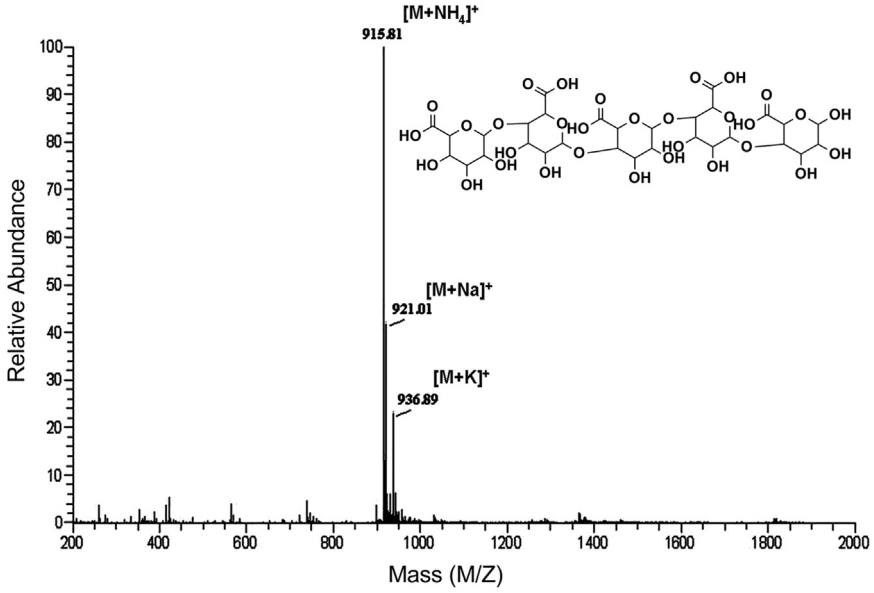

Fig. 1. Chemical structure of AOG.

2.9. Murine model of xenograft tumor and treatment

Thirty male BALB/c nude mice aged 5 weeks were maintained in pathogen-free conditions. HT-29 cells (5×10^6) were injected subcutaneously into the right flank of nude mice. Treatments (6 mice per group) were started on the 4th day after HT-29 cells injections. Control diluents, 5-FU (20 mg/kg), AOG (20 mg/kg) and 5-FU (20 mg/kg) plus AOG (20 mg/kg) were administered through tail intravenous injection every three days till the end of experiment (25 days). Tumor volumes were calculated by the formula: $0.5\times a\times b^2$ in millimeters, in which 'a' is the length and 'b' is the width.

2.10. Western blot analysis

The procedure was carried out as previously described (Li et al., 2009). Protein lysates were obtained, electrophoresed and transferred to nitrocellulose membranes. The signals were detected using the Lumigen TMA-6 kit (GE, UK).

2.11. TUNEL immunohistochemical assay

The DeadEnd™ Colorimetric TUNEL System (Promega, San Luis Obispo, CA, USA) was used for detection of apoptotic cells according to the manufacturer's instructions. Frozen sections were fixed with 4% paraformaldehyde and incubated with the terminal deoxynucleotidyl transferase (TdT) buffer. Stained sections were examined under a Nikon microscope (Tokyo, Japan).

2.12. Immunohistochemical study

Immunohistochemistry for Ki-67 was performed on 4-μm-thick paraffin-embedded sections from colons of mice. After microwave antigen retrieval, slides were incubated with 5% normal goat serum for 20 min in a humidified chamber to reduce non-specific binding, and then incubated overnight at 4°C with antibody to Ki-67 (1:300). After successive PBS washes (three times, 5 min each), slides were incubated with streptavidin-peroxidase complex and then visualized using 3,3′-diaminobenzidine tetrahydrochloride. Stained sections were examined under a Nikon microscope.

2.13. NF-κB transcription factor assay

NF-κB p65 subunit DNA binding activity was determined by an enzyme-linked immunosorbent assay (Cayman Chemicals, Ann Arbor, MN, USA) according to the manufacturer's instructions. In brief, a specific doublestranded DNA sequence containing the NF-κB (p65) response element was immobilized onto the bottom of wells of a 96-well plate. Nuclear extracts were added to the plate and incubated overnight at 4 °C. NF-κB (p65) was detected by addition of a primary antibody against p65.

2.14. Statistical analysis

Statistical differences among the control, model, and different treatment groups were analyzed using one-way analysis of variance (ANOVA) followed by a post hoc multiple comparisons using Fisher's least significant difference (LSD) *t*-test. The differences of the ratio between two groups were detected by chi-square test. Probability (*P* value) of less than 0.05 was considered to be statistically significant.

3. Results

3.1. AOG enhanced the growth inhibitory effect of 5-FU in vitro

The IC_{50} values of 5-FU were 26.70 ± 0.21 μM in HT-29 cells and 26.71 ± 2.06 μM in SW-620 cells (and 20.67 ± 1.54 μM in HCT-116 cells, data not shown). Interestingly, pretreatment with 0.05 or 0.1 mM AOG reduced IC_{50} values of 5-FU to 22.44 ± 1.01 or 18.67 ± 1.16 μM in HT-29 and 21.21 ± 1.49 or 17.99 ± 1.42 μM in SW-620 cells (and 15.16 ± 0.96 μM or 12.46 ± 1.01 μM in HCT-116 cells). To exclude the inhibitory effect caused by the cytotoxic effect of AOG, a normal human intestinal epithelial cell line (H-IEC) was treated with various concentrations (0.01–1 mM) of AOG for 24 h, and the cell viability was determined. The results showed that AOG had no significant cytotoxicity on H-IEC, even at the concentration of 1 mM (Fig. 2). And AOG hardly affected the growth inhibitory effect of 5-FU in H-IEC (data not shown). Based on the IC_{50} value, the concentrations of 0.05 mM or 0.1 mM AOG plus 15 μM 5-FU were chosen in the following steps.

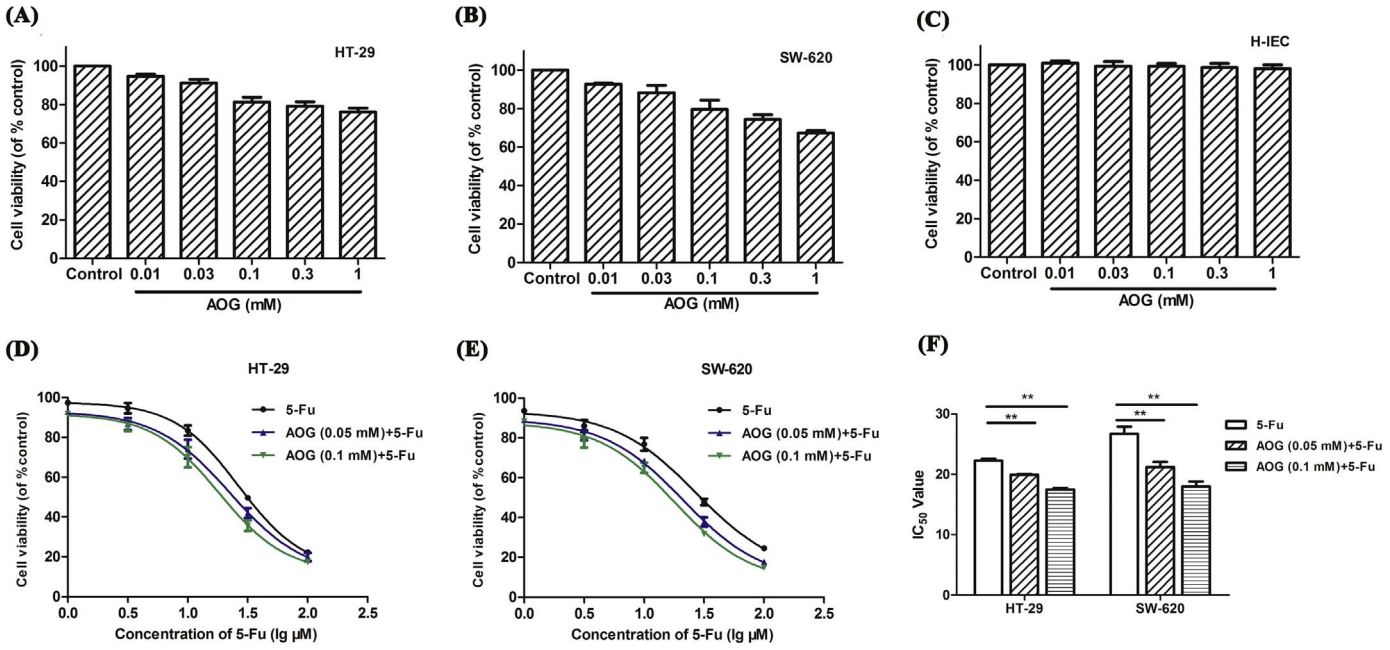

Fig. 2. The effect of AOG, 5-FU or their combination on the growth of human colorectal cancer cell lines. (A), (B), (C) The effect of AOG at the concentrations of 0.01, 0.03, 0.1, 0.3, 1 mM on HT-29 cells, SW-620 cells or a normal human intestinal epithelial cell line H-IEC. (D), (E) The effect of 5-FU at the concentrations of 1, 3, 10, 30, 100 μM or 5-FU plus 0.05, 0.1 mM AOG on HT-29, or SW-620 cells. (F) The IC_{50} values of HT-29 and SW-620 cells. The IC_{50} values of 5-FU were 26.70 ± 0.21 μM in HT-29 cells and 26.71 ± 2.06 μM in SW-620 cells. Pretreatment with 0.05 or 0.1 mM AOG down-regulated IC $_{50}$ values of 5-FU to 22.44 ± 1.01 or 18.67 ± 1.16 μM in HT-29 and 21.21 ± 1.49 or 17.99 ± 1.42 μM in SW-620 cells. Each column represents the mean with S.D. in triplicates in three independent experiments.

3.2. The combinatorial therapy triggered cell apoptosis and induced cell cycle arrest at S phase

The percentage of apoptotic cells (B2+ B4) in HT-29 and SW-620 cells was 3.3 ± 0.6% and 2.6 ± 0.9%. When exposed to 5-FU, AOG, and 5-FU plus AOG for 24 h, the percentage increased to 12.5 ± 1.7%, 6.7 ± 1.2%, 9.6 ± 1.6%, 16.3 ± 1.1% and 26.3 ± 3.2% in HT-29 cells respectively; and the percentage increased to 15.8 ± 1.2%, 8.8 ± 0.8%, 12.4 ± 0.7%, 19.3 ± 0.8% and 33.5 ± 2.9% in SW-620 cells (Fig. 3A).

The analysis of nuclear DNA distribution revealed an increased accumulation of S phase cells, compared with those in the control group (Fig. 3B).

3.3. The combinatorial therapy affected expression of apoptosis and cell cycle-related proteins

After 24 h of treatment, caspase-3 was cleaved into its active form. In parallel to the alteration, expression of cleaved-caspase-9 was increased; Cleaved caspase-8 expression, however, had no obvious changes. The combination significantly up-regulated the expression of Apaf-1 and Cyto c. We next investigated the expression of Bcl-2 families, which regulate mitochondrial apoptosis and could be separated into pro-survival members (such as Bcl-2, BclxL, and Mcl-1), as well as pro-apoptotic proteins (such as Bax) (Cory et al., 2002; Kroemer et al., 1997). As shown in Fig. 4a, after different treatments, Bcl-2 was down-regulated significantly, while Bax was up-regulated on the contrary, especially in combinatorial treatment group (Fig. 4A). The activity of caspase-3 was also measured. As shown in Fig. 5a, obvious activation of caspase-3 was observed in HT-29 and SW-620 cells treated with 5-FU, AOG, or their combination. The increased activity of caspase-3 was significantly blocked by pretreatment of a general caspase inhibitor, Z-VAD-FMK (20 μM) (Fig. 4B). These data suggest that combinatorial treatment induced apoptosis of HT-29 and SW-620 cells possibly through an intrinsic pathway that depended on caspase activation.

To investigate the mechanisms involved in the S phase arrest, the expression of Cyclin A and CDK-2 was evaluated. 5-FU, AOG and their combination decreased the expression of Cyclin A as well as CDK-2. Co-immunoprecipitation assay revealed that Cyclin A and CDK-2 were still found to interact after treatment with AOG. However, their binding capacity was reduced (Fig. 4A, C).

3.4. The combination protected ICR mice against intestinal toxicities and carcinogenesis induced by DMH/DSS

Table 1 summarizes the ability of 5-FU, AOG or their combination to protect against intestinal toxicity and reduce colon tumor development in the DMH/DSS-treated ICR mice. After DMH/DSS treatment, piloerection, bowing waist, and loose stool were observed in nearly all the mice. 5-FU, AOG or the combination were initiated in the 12th week, when colons of DMH/DSS-treated mice showed a sign of carcinogenesis. 5-FU, AOG or the combination effectively protected ICR mice against DMH/DSS-induced carcinogenesis. The incidence of tumor formation was 90% (9/10) in the mice treated with DMH/DSS, and it was then reduced to 20% (2/10), 30% (3/10), and 0% (0/10) with DMH/DSS plus 5-FU, AOG or the combination. It was worth mentioning that 5-FU plus AOG showed the most potent effect in preventing CACC (Table 1 and Fig. 5A).

The representative data of pathological examinations are shown in Fig. 6. The normal histomorphology of the colon from the control illustrated integral epithelial cell structure (Fig. 6B). Neoplastic glands (cribriform pattern) developed with marked cytological atypia and mitotic activity of the colon in the 20th week after DMH/DSS treatment, and only lymphocytic infiltrate in the mucosa and edema in the submucosa of the colons were observed after the treatment of 5-FU, AOG or their combination (Fig. 5B). The Ki-67 protein is a proliferation antigen. Quiescent or resting cells in the G_0 phase of the cell cycle do not express the Ki-67 antigen. Since it is not involved in DNA repair process as PCNA, the Ki-67 index may be the best available marker of proliferation (Vilar et al., 2007). As shown in Fig. 5c, notable reduction of proliferation (Ki-67 staining) was observed in combination-treated ICR mice. Results of TUNEL staining showed that 5-FU, AOG or their combination induced apoptosis of colonic epithelial cells (Fig. 5D). The combinatorial treatment presented the most obvious apoptosis inducing effect.

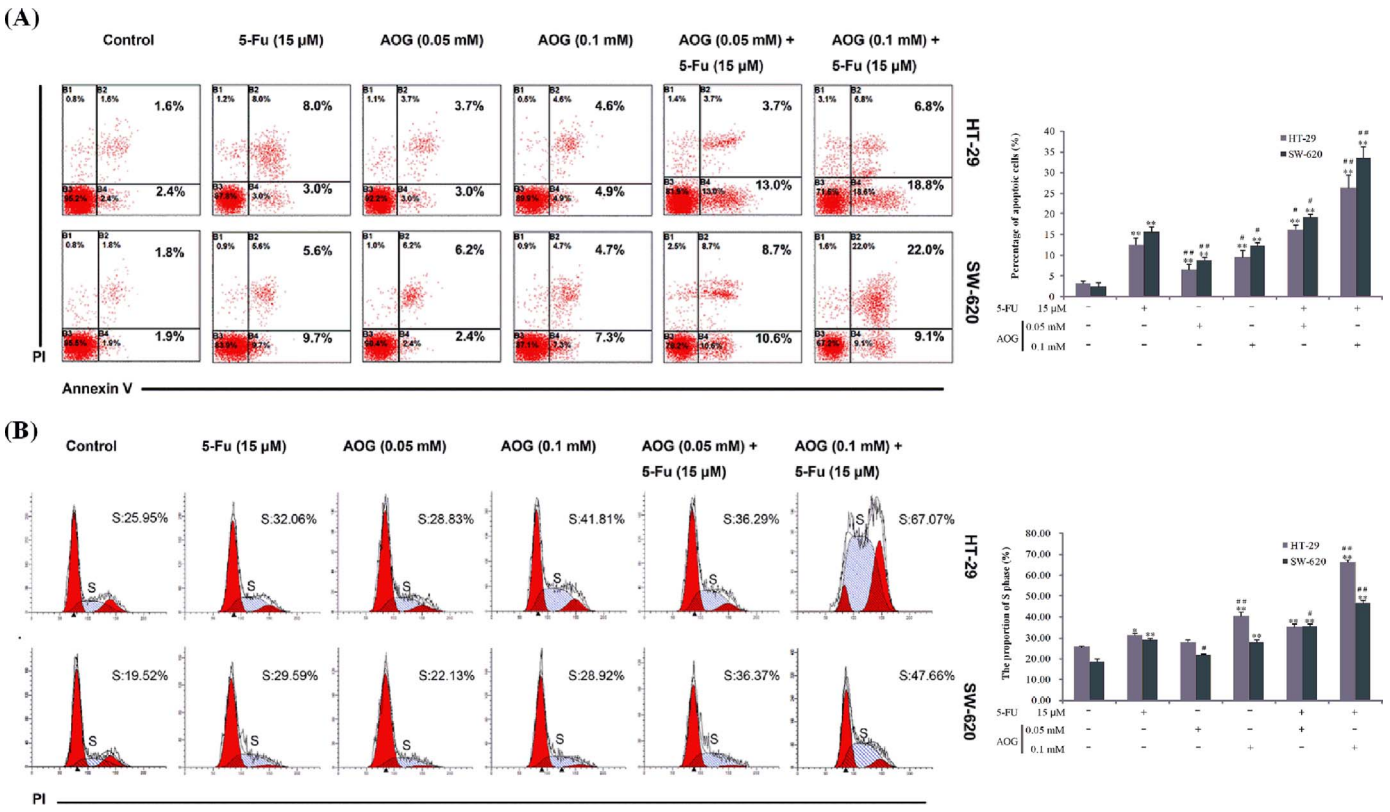

Fig. 3. The effect of 5-FU, AOG or their combination on apoptosis and cell cycle of HT-29 and SW-620 cell lines. (A) Representative results of Annexin V analysis of HT-29 and SW-620 cells treated with 5-FU, AOG or their combination for 24 h. The treatments increased the percentages of annexin-V^+/PI^+(down right and up right quadrants) cells. (B) Characteristic contribution of nuclear DNA content in HT-29 and SW-620 cells treated with 5-FU, AOG or their combination for 24 h. Significant increase of S cells was observed. The experiments were repeated independently thrice.

3.5. In vivo effect of the combination on xenograft model of human colorectal cancer

We next performed intravenus injection of 5-FU, AOG and their combination to confirm their anti-tumor effect in vivo, by using a subcutaneous xenograft model. As shown in Fig. 6A-D, treatment of 5-FU, AOG and their combination significantly suppressed tumor growth. The combinatorial treatment demonstrated the most potent effect. Ki-

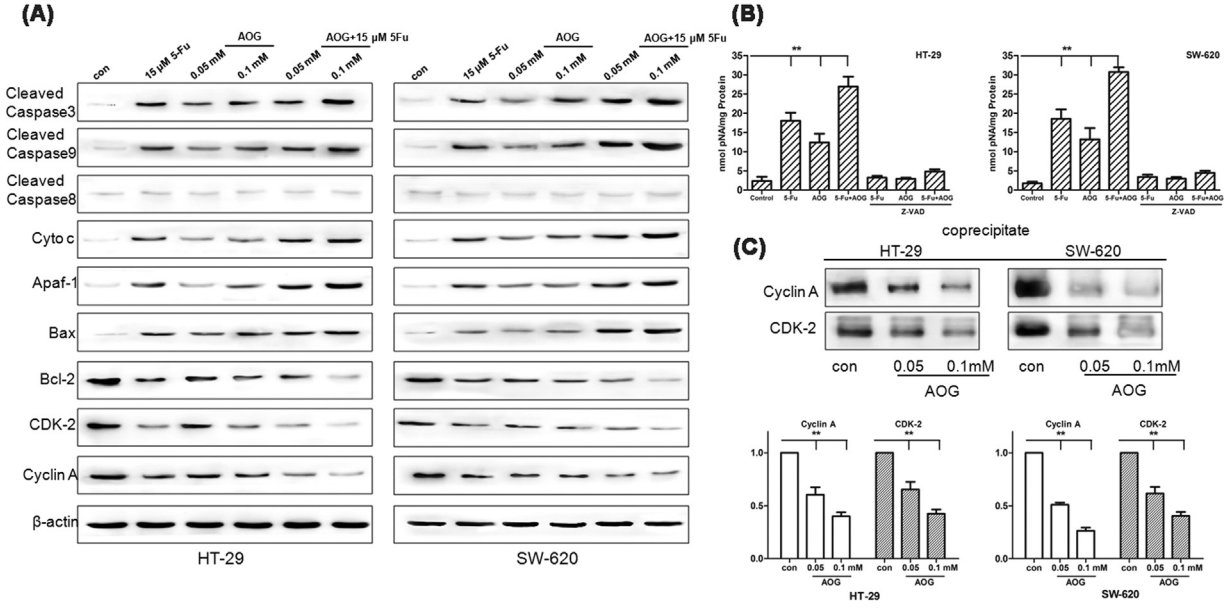

Fig. 4. The effect of 5-FU, AOG or their combination on apoptosis related, cell cycle related proteins expression and the caspase-3 activity, and the effect of AOG on the interaction between Cyclin A and CDK-2 of HT-29 and SW-620 cell lines. (A) 5-FU, AOG or their combination increased cleaved caspase 3, 9, Cyto-c, Apaf-1and Bax, while reduced Bcl-2, Cyclin A and CDK-2, and had no significant effect on cleaved caspase 8 expressions in protein level in HT-29 and SW-620 cells. The results are representative of three repeats. (B) Effect of AOG on the caspase-3 activity of HT-29 and SW-620 cells. Data were the mean ± S.D. of three separate experiments. **P < 0.01 vs. control. (C) Western blot analysis of binding between Cyclin A and CDK-2 in cells after AOG treatments. 500 μg of extracted total proteins was co-immunoprecipitated (Co-IP) with 3 μg of anti-Cyclin A antibody. The Co-IP products were resuspended in an equal volume of loading buffer, and the supernatant was subjected to western blot with anti-Cyclin A and anti-CDK-2.

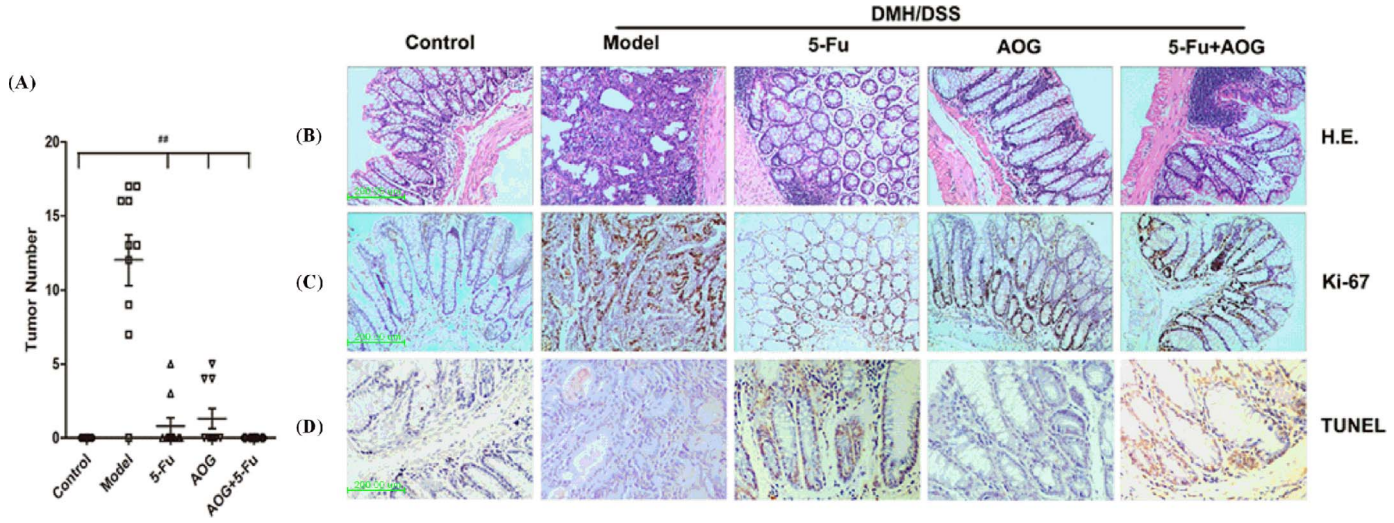

Fig. 5. The effect of 5-FU, AOG or their combination on the tumorigenesis of DMH/DSS-treated ICR mice. (A) The tumor number in the intestine of the mice. The mice were given 5-FU (20 mg/kg), AOG (20 mg/kg) or AOG (20 mg/kg) plus 5-FU (20 mg/kg). Each dot represents one mouse; bars, mean ± S.D.; n =10 (B) H.E. staining of colon in mice from the groups of control, DMH/DSS, DMH/DSS+5-FU, DMH/DSS+AOG, and DMH/DSS+AOG+5-FU (100× magnification). (C) Ki-67 staining of colon in mice from the groups of control, DMH/DSS, DMH/DSS+5-FU, DMH/DSS+AOG, and DMH/DSS+AOG+5-FU (200× magnification). (D) TUNEL staining of colon in mice from the groups of control, DMH/DSS, DMH/DSS+5-FU, DMH/DSS+AOG, and DMH/DSS+AOG+5-FU (200× magnification). Ten mice were measured in each group.

67 staining also revealed notable reduction of proliferation in combination-treated tumor xenograft (Fig. 6E). Results of TUNEL staining showed that 5-FU, AOG and the combination could induce cellular apoptosis in tumor (Fig. 6F). The combination presented the most obvious apoptosis inducing effect. The effects of the combination on apoptosis-related and cell cycle-related proteins expression were similar to those of in vitro (Fig. 6G).

3.6. The combination suppressed NF-κB signals

We further detected the inhibitory effect of the 5-FU, AOG or their combination on NF-κB activity. As shown in Fig. 7A, NF-κB DNA binding activity was dramatically decreased after the treatment for 6 h ($P < 0.05$). Moreover, 5-FU, AOG or their combination also induced the dephosphorylation and up-regulation of IκBa, as well as the suppression of NF-κB translocation into nucleus (Fig. 7B). In comparison to 5-FU alone, AOG or their combination showed a more potent effect in reducing the expression of TLR-4, an important upstream molecule of NF-κB, in the cell membrane (Fig. 7C).

4. Discussion

5-FU is routinely used for the management of patients with CRC (Borralho et al., 2007). However, the long-term use of 5-FU as a CRC chemotherapeutic agent may be limited due to the toxicities, limited success and associated adverse side effects. Meanwhile, a growing number of evidence indicates that natural occurring substances as dietary supplements could reduce cancer risk and hold a central role in the development of anti-tumor drugs (Guptam et al., 2010; Newman et al., 2003). Indeed emerging literature suggests that conventional chemotherapy in CRC significantly benefits from combinatorial treatments with some of such naturally occurring dietary components. It has been reported that Curcumin and FOLFOX synergistically eliminate colon cancer stem-like cells (Yu et al., 2009). Shikonin, a natural inducer of ROS, enhances cisplatin-induced colon cancer cells apoptosis in vitro and in vivo (He et al., 2016). Since combinatorial strategies could be more effective and much safer than monotherapy in cancer treatment, they arouse clinical and scientific interest.

In current study, we found that AOG alone or in combination with 5-FU significantly suppressed proliferation, induced apoptosis and cell

Table 1
The effects of 5-Fu, AOG and their combination on bloody stool, anal prolapse, colon tumor formation, colon length, 4 cm Colon weight, and body weight in mice treated with DMH/DSS.

Group	Bloody stool		Anal prolapse		Colon tumor	Colon length (cm)	4 cm Colon weight (g)	Body weight (g)
	Time							
	Week 3	Week 9	Week 12	Week 15	Week 20	Week 20	Week 20	Week 20
Control (Saline)	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	10.52 ± 0.50	0.16 ± 0.02	45.59 ± 3.00
DMH/DSS	31/40 (78%)	20/40 (50%)	6/10 (60%)	9/10 (90%)	9/10 (90%)	8.49 ± 0.36[a]	0.61 ± 0.06 [a]	32.27 ± 5.38 [a]
DMH/DSS+5-Fu(20 mg/kg)	-	-	2/10 (20%)	1/10 (10%)	2/10 (20%)	9.71 ± 0.44[c]	0.32 ± 0.05[c]	43.90 ± 3.62[b]
DMH/DSS+AOG(20 mg/kg)	-	-	2/10 (20%)	3/10 (30%)	3/10 (30%)	9.57 ± 0.59[c]	0.39 ± 0.10[c]	42.31 ± 4.53[b]
DMH/DSS+5-Fu(20 mg/kg)+AOG(20 mg/kg)	-	-	1/10 (10%)	0/10 (0%)	0/10 (0%)	10.25 ± 0.63[c]	0.18 ± 0.03[c]	45.01 ± 2.83[b]

A total of 50 mice were used for the experiment, 10 mice were used as control, 10 mice for DMH/DSS, 10 mice for DMH/DSS +5-Fu, 10 mice for DMH/DSS + AOG, and DMH/DSS +5-Fu + AOG. The mice in 5-Fu, AOG and their combination groups were administered through tail intravenous injection with 5-Fu, AOG or their combination every four days, from the 12th week to the end of experiment (20th week). Data were expressed as mean ± standard deviation (S.D.). [a]$P < 0.01$ versus Control. [b]$P < 0.05$ versus DMH/DSS, [c]$P < 0.01$ versus DMH/DSS.

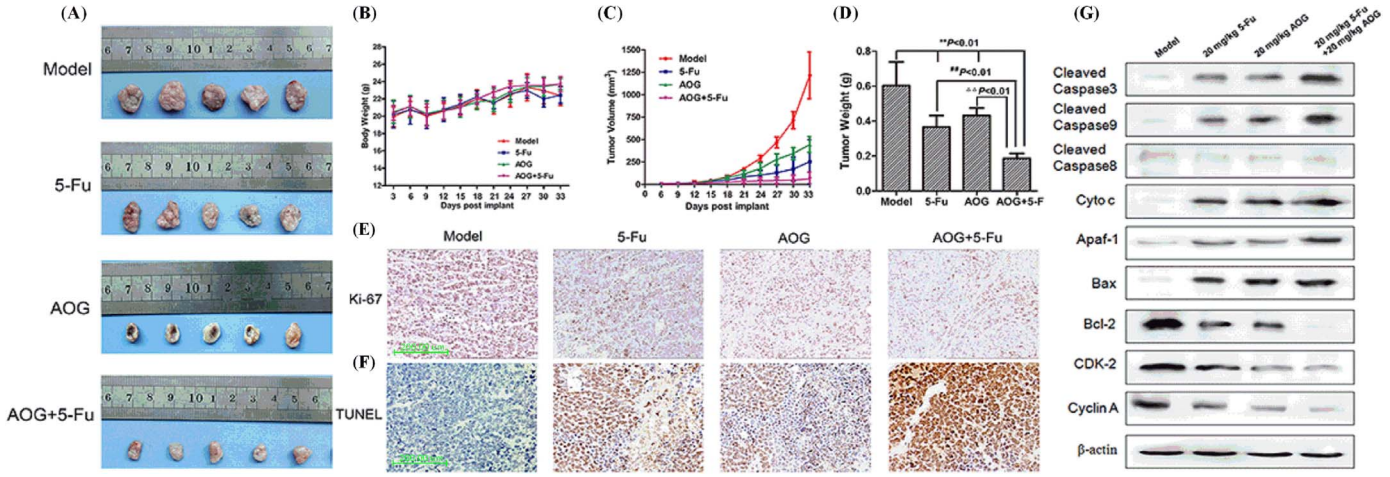

Fig. 6. The effect of 5-FU, AOG or their combination on murine models of HT-29 cells. (A) General observation of xenograft tumor at the end of the experiment. Nude mice were injected with 5×10^6 of HT-29 cells (Day 0) in nude mice. The mice were given 5-FU (20 mg/kg), AOG (20 mg/kg) or 5-FU (20 mg/kg) plus AOG (20 mg/kg) through tail intravenous injection every three days from Day 4 to the end of experiment (25 days). 5-FU, AOG or their combination decreased the size of tumor (Day 25). (B) Effect of 5-FU, AOG or their combination on the body weight of nude mice. (C) 5-FU, AOG or their combination could significantly reduce the xenograft tumor volumes ($P < 0.01$). Each point represents the mean with S.D. of 6 tumors. (D) 5-FU, AOG or their combination could significantly decrease the xenograft tumor weights ($P < 0.01$). (E) Ki-67 staining was used to detect the proliferate level of tumor cells. (F)TUNEL staining was used to determine the level of apoptosis. (G) 5-FU, AOG or their combination increased cleaved caspase 3, 9, Cyto-c, Apaf-1and Bax, while reduced Bcl-2, Cyclin A and CDK-2, and had no significant effect on cleaved caspase 8 expressions in tumor tissues (n=5).

cycle arrest in CRC cells, and showed a growth inhibitory effect on a murine model of xenograft tumor. In addition, AOG alone or in combination with 5-FU demonstrated a potential protective efficacy on intestinal toxicities and carcinogenesis in DMH/DSS-induced CACC mouse model.

The combination decreased cell viability partly through the induction of apoptosis. Protein levels of cleaved-caspase-9, Apaf-1, Cyto-c and Bax were significantly up-regulated, while cleaved-caspase-8 had no significant changes, suggesting the apoptosis induced by the combination was closely related to intrinsic mitochondrial apoptotic pathway. Bcl-2, as a major anti-apoptotic protein of Bcl-2 family (Cory et al., 2003; Kroemer, 1997), was greatly reduced by the combination, further indicating that intrinsic pathway was involved.

Combinatorial treatment-induced S phase arrest may be another reason for the inhibition of cell growth. Cyclin A binds to CDK-2 and this complex is required during S phase (Girard et al., 1991; Walker

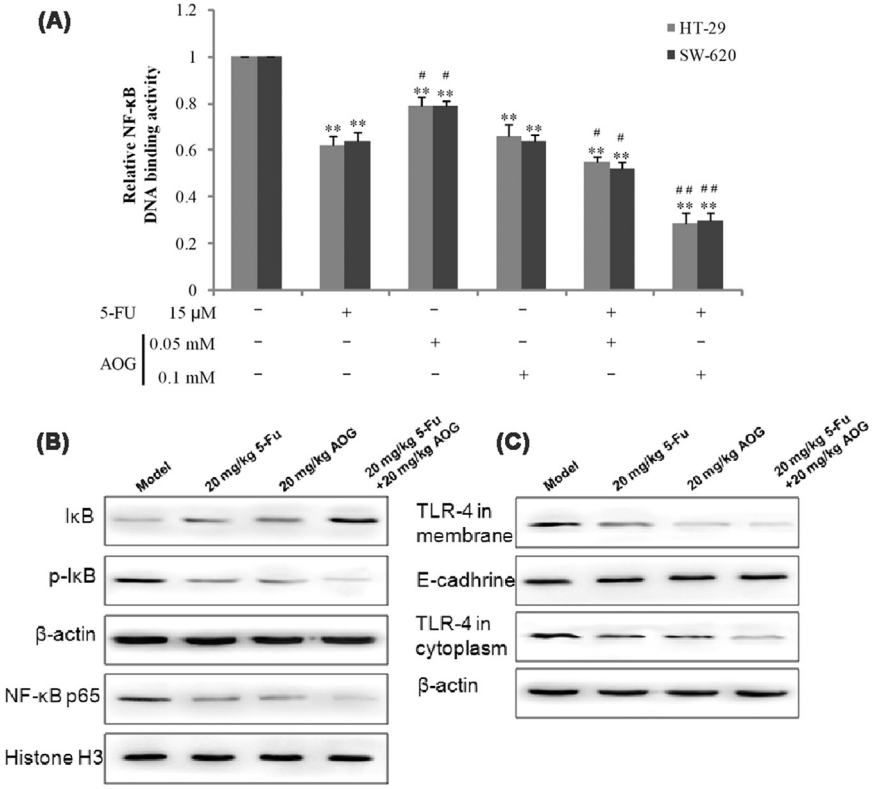

Fig. 7. The effect of 5-Fu, AOG or their combination on NF-κB DNA binding activity in HT-29 and SW-620 cells, and IκBα phosphorylation and degradation, and the distribution of TLR-4 on murine models of HT-29 cells (n=5). (A) NF-κB DNA binding activity after 5-Fu, AOG or their combination for 6 h was determined by using an ELISA assay. *$P < 0.05$, and **$P < 0.01$ vs. control. #$P < 0.05$, and ##$P < 0.01$ vs. 5-FU treatment. (B) Whole-cell lysates or nucleic extracts of tumor tissue of nude mice HT-29 were prepared respectively, and immunoblotted with specific antibodies against IκBα, phosphorylated IκBα, p65, β-actin, and histon H3 (C) The effect of 5-Fu, AOG or their combination on protein level of TLR-4.

and Maller, 1991). Cyclin A and CDK-2 expressions were significantly down-regulated by the combination. AOG could reduce the expression of Cyclin A and CDK-2 as well as their binding capacity, at least partly explaining why AOG could enhance the effect of 5-FU. Though AOG just showed a moderate growth inhibitory effect, it could induce CRC cells arrest in S phase effectively. Considering that 5-FU mainly affects tumor cells in S phase, it seems to be reasonable that pretreated with AOG would enhance the effect of 5-FU. Indeed, we have found that the most potent growth inhibitory effect did appear when AOG was used before rather than when it was used together with or behind 5-FU treatment (data not shown).

The NF-κB pathway plays an important role in tumorigenesis through transactivation of genes involved in cell proliferation and apoptosis (Orlowski and Baldwin, 2002). It is supported by previous research that several NF-κB inhibitors, such as BAY 11−7082, BMS-345541 and PS-1145 could decrease the expression of Cyclin A and CDK-2 in gastric cancer cells or activated $CD4^+$ T cells (Chen et al., 2014; Lupino et al., 2010), implicating the critical role of NF-κB pathway in modulation of Cyclin A and CDK-2 expression. Tumor cells may also rely on the NF-κB pathway to escape from apoptosis. Activation of NF-κB inhibits apoptosis by upregulating the expression of Bcl-2 family members and caspases inhibitor (Lin and Karin, 2007). Our study demonstrated that AOG effectively inhibited NF-κB activation through preventing the degradation of IκB, suppressing the phosphorylation of IκB, and blocking the translocation of NF-κB (p65). TLR-4 is an important upstream molecule of NF-κB; TLR-4/NF-κB pathway plays a pivotal role in the initiation and growth of CRC (Fukata et al., 2007; Greten et al., 2004; Rakoff-Nahoum and Medzhitov, 2007). The expression of TLR-4 at the cell surface in enterocytes and macrophages could bind and internalize with its ligands such as LPS to trigger the downstream signaling pathway including NF-κB pathway (Wakabayashi et al., 2006). In current experiment, we found that compared with 5-FU alone, AOG or their combination could significantly decrease the expression of TLR-4 in the cell membrane. Taken all the results together, we assume that AOG first suppresses TLR-4/NF-κB pathway, then, induces S phase arrest and apoptosis, finally potentiates the effect of 5-FU.

In conclusion, AOG enhances the growth inhibitory effect of 5-FU. The possible mechanism may be that AOG triggers S phase arrest and apoptosis of CRC cells through the suppressiones of TLR-4/NF-κB pathway. Although more CRC cell lines and animal models are needed to confirm the effect, these data suggest that the combinatorial therapy using AOG and 5-FU is a promising strategy for the treatment of colorectal cancer.

Acknowledgements

This investigation was supported by the grant [No. 81302787], from National Nature Science Foundation of China and the grant [No. 2013T60964] from Postdoctoral Science Foundation of China and the grant [No. 2016JQ8005] from the Natural Science Foundation Research Project of Shaanxi Province and the grant [No. 15JK1636] from the Education Department of Shaanxi Province.

References

American Cancer Society, 2013. Cancer Facts & Figures 2013. American Cancer Society, Atlanta.

Barth, S.W., Fähndrich, C., Bub, A., et al., 2005. Cloudy apple juice decreases DNA damage, hyperproliferation and aberrant crypt foci development in the distal colon of DMH-initiated rats. Carcinogenesis 26, 1414–1421.

Borralho, P.M., Moreira, da Silva, I.B., Aranha, M.M., et al., 2007. Inhibition of Fas expression by RNAi modulates 5-fluorouracil-induced apoptosis in HCT116 cells expressing wild-type p53. Biochim. Biophys. Acta 1772, 40–47.

Center, M.M., Jemal, A., Smith, R.A., et al., 2009. Worldwide variations in colorectal cancer. CA Cancer J. Clin. 59, 366–378.

Chen, L., Ruan, Y., Wang, X., et al., 2014. BAY 11-7082, a nuclear factor-κB inhibitor, induces apoptosis and S phase arrest in gastric cancer cells. J. Gastroenterol. 49, 864–874.

Cory, S., Adams, J.M., 2002. The Bcl2 family: regulators of the cellular life-or-death switch. Nat. Rev. Cancer 2, 647–656.

Divisi, D., Di, Tommaso, S., Salvemini, S., et al., 2006. Diet and cancer. Acta Biomed. 77, 118–123.

Fukata, M., Chen, A., Vamadevan, A.S., et al., 2007. Toll-like receptor-4 promotes the development of colitis-associated colorectal tumors. Gastroenterology 133, 1869–1881.

Gerhauser, C., 2008. Cancer chemopreventive potential of apples, apple juice, and apple components. Planta Med. 74, 1608–1624.

Girard, F., Strausfeld, U., Fernandez, A., et al., 1991. Cyclin A is required for the onset of DNA replication in mammalian fibroblasts. Cell 67, 1169–1179.

Greten, F.R., Eckmann, L., Greten, T.F., et al., 2004. IKKbeta links inflammation and tumorigenesis in a mouse model of colitis-associated cancer. Cell 118, 285–296.

Guptam, S.C., Kim, J.H., Prasad, S., et al., 2010. Regulation of survival, proliferation, invasion, angiogenesis, and metastasis of tumor cells through modulation of inflammatory pathways by nutraceuticals. Cancer Metastas. Rev. 29, 405–434.

He, G., He, G., Zhou, R., et al., 2016. Enhancement of cisplatin-induced colon cancer cells apoptosis by shikonin, a natural inducer of ROS in vitro and in vivo. Biochem. Biophys. Res. Commun. 469, 1075–1082.

He, X., Liu, R.H., 2008. Phytochemicals of apple peels: isolation, structure elucidation, and their antiproliferative and antioxidant activities. J. Agric. Food Chem. 56, 9905–9910.

Hogg, N., 2007. Red meat and colon cancer: heme proteins and nitrite in the gut. A commentary on "diet-induced endogenous formation of nitroso compounds in the GI tract". Free Radic. Biol. Med. 43, 1037–1039.

Jedrychowski, W., Maugeri, U., Popiela, T., et al., 2010. Case-control study on beneficial effect of regular consumption of apples on colorectal cancer risk in a population with relatively low intake of fruits and vegetables. Eur. J. Cancer Prev. 19, 42–47.

Jedrychowski, W., Maugeri, U., 2009. An apple a day may hold colorectal cancer at bay: recent evidenc from a case-control study. Rev. Environ. Health 24, 59–74.

Jemal, A., Bray, F., Center, M.M., et al., 2011. Global cancer statistics. CA Cancer J. Clin. 61, 69–90.

Kroemer, G., 1997. The proto-oncogene Bcl-2 and its role in regulating apoptosis. Nat. Med. 3, 614–620.

Li, Y., Niu, Y., Wu, H., et al., 2009. PC-407, a celecoxib derivative, inhibited the growth of colorectal tumor in vitro and in vivo. Cancer Sci. 100, 2451–2458.

Lin, W.W., Karin, M.A., 2007. cytokine-mediated link between innate immunity, inflammation, and cancer. J. Clin. Investig. 117, 1175–1183.

Liu, L., Li, Y.H., Niu, Y.B., et al., 2010. An apple oligogalactan prevents against inflammation and carcinogenesis by targeting LPS/TLR4/NF-κB pathway in a mouse model of colitis-associated colon cancer. Carcinogenesis 31, 1822–1832.

Lokich, J.J., Ahlgren, J.D., Gullo, J.J., et al., 1989. A prospective randomized comparison of continuous infusion fluorouracil with a conventional bolus schedule in metastatic colorectal carcinoma: a Mid-Atlantic Oncology Program Study. J. Clin. Oncol. 7, 425–432.

Lupino, E., Buccinnà, B., Ramondetti, C., Lomartire, A., De Marco, G., Ricotti, E., Tovo, P.A., Rinaudo, M.T., Piccinini, M., 2010. In CD28-costimulated human naïve CD4+ T cells, I-κB kinase controls the expression of cell cycle regulatory proteins via interleukin-2-independent mechanisms. Immunology 131, 231–241.

Meta-analysis Group In Cancer, Piedbois, P., Rougier, P., et al., 1998. Efficacy of intravenous continuous infusion of fluorouracil compared with bolus administration in advanced colorectal cancer. J. Clin. Oncol. 16, 301–308.

Newman, D.J., Cragg, G.M., Snader, K.M., 2003. Natural products as sources of new drugs over the period 1981–2002. J. Nat. Prod. 66, 1022–1037.

Orlowski, R.Z., Baldwin, A.S., Jr., 2002. NF-kappaB as a therapeutic target in cancer. Trends Mol. Med. 8, 385–389.

Rakoff-Nahoum, S., Medzhitov, R., 2007. Regulation of spontaneous intestinal tumorigenesis through the adaptor protein MyD88. Science 317, 124–127.

Vilar, E., Salazar, R., Pérez-García, J., et al., 2007. Chemotherapy and role of the proliferation marker Ki-67 in digestive neuroendocrine tumors. Endocr. Relat. Cancer 14, 221–232.

Wakabayashi, Y., Kobayashi, M., Akashi-Takamura, S., et al., 2006. A protein associated with toll-like receptor 4 (PRAT4A) regulates cell surface expression of TLR4. J. Immunol. 177, 1772–1779.

Walker, D.H., Maller, J.L., 1991. Role for cyclin A in the dependence of mitosis on completion of DNA replication. Nature 354, 314–317.

Yoon, H., Liu, R.H., 2007. Effect of selected phytochemicals and apple extracts on NF-kappaB activation in human breast cancer MCF-7 cells. J. Agric. Food Chem. 55, 3167–3173.

Yu, Y., Kanwar, S.S., Patel, B.B., et al., 2009. Elimination of colon cancer stem-Like cells by the combination of Curcumin and FOLFOX. Transl. Oncol. 2, 321–328.