

# Combination of rapamycin and garlic-derived S-allylmercaptocysteine induces colon cancer cell apoptosis and suppresses tumor growth in xenograft nude mice through autophagy/p62/Nrf2 pathway

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**Abstract.** The natural plant-derived product S-allylmercaptocysteine (SAMC) has been studied in cancer therapy as a single and combination chemotherapeutic agent. The present study was employed to verify the combination use of SAMC and rapamycin that is the mTOR inhibitor with anticancer ability but has limited efficacy due to drug resistance, and to explore the underlying mechanisms. We combined rapamycin and SAMC for colorectal cancer treatment in the HCT-116 cancer cells and a xenograft murine model. The *in vivo* study was established by xenografting HCT-116 cells in BALB/c nude mice. It was found that the combination therapy had enhanced tumor-suppressing ability with the upregulation of the Bax/Bcl-2 ratio as a consequence of activated apoptosis, inhibition of autophagic activity and prevention of Akt phosphorylation. The rapamycin and SAMC combination activated antioxidant transcription expressions of Nrf2 and downstream gene NQO1. Concomitantly, autophagosome cargo p62 was downregulated, indicating that the p62 played a negative-regulatory role between Nrf2 and autophagy. Our results show that the combination of SAMC and rapamycin enhanced the anticancer ability, which could be used for the treatment of colorectal cancer. The underlying mechanism of autophagy/p62/Nrf2 pathway discovered may provide

a new direction for drug development, especially for traditional Chinese medicines.

## Introduction

Globally, colorectal cancer (CRC) is the second and third most common causes of cancer in women (9.2% of diagnoses) and men (10.0%), respectively (1). Colorectal cancer death ranks fourth after lung, stomach, and liver cancer, which is more common in developed countries (2). Treatments used for colorectal cancer include rational combination of surgery, radiation therapy, chemotherapy and targeted therapy.

In recent years, autophagy has become a topic of debate in cancer (3). Autophagy is a conserved lysosomal degradation process of intracellular recycling and degraded metabolites, and can maintain the cellular homeostasis and adaption to stress conditions. In cancer, autophagy plays a complex role in tumor initiation and growth. It can either induce cancer cell death by eliminating carcinogenic factors and damaged organelles in the initial stage of cancer; or, promote cancer cell survival by intracellular recycling of degraded metabolites to cope with starvation or stress conditions in cancer progression (4). In addition, autophagy may contribute to drug resistance due to an adaptive response to chemotherapy and radiation therapy (5).

The mammalian target of rapamycin (mTOR) signaling plays a role in suppressing autophagy (6). The dysregulation of the mTOR activity is found linking to the initiation and development of many human tumors (7). The most established mTOR inhibitors such as rapamycin have been evaluated as anticancer agents, and used in the treatment of cancer in clinical trials (8).

Rapamycin inhibits mTOR by binding the FK506 binding protein 1A, 12 kDa (FKBP-12) (9). However, the use of mTOR inhibitors is limited because of the occurrence of cancer escape due to drug resistance. The mechanism is related to oncogene Akt reactivation by negative mTOR-PI3K feedback (10).

The use of natural plant-derived products in cancer therapy has gained attention in recent years. Garlic, a plant within the

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*Abbreviations:* SAMC, S-allylmercaptocysteine; mTOR, mammalian target of rapamycin; OSCs, organosulfur compounds; Nrf2, nuclear factor (erythroid-derived 2)-like 2; HO-1, heme oxygenase-1; NQO1, NAD(P)H: quinone oxidoreductase 1; TCM, traditional Chinese medicine

*Key words:* rapamycin, S-allylmercaptocysteine, autophagy, Nrf2, colorectal cancer, p62

genus *Allium*, has been explored by focusing on many organo-sulfur compounds (OSCs). S-allylmercaptocysteine (SAMC), the water-soluble fraction, is one of the major part in OSCs (11). SAMC has been studied in various cancer cells for its antiproliferative effect (12,13), and *in vivo* studies also showed tumor suppressive ability (14,15). Moreover, SAMC is a good adjuvant in combination with chemopreventive agents (16).

The relationship between autophagy and Nrf2 pathway has been studied, promoting a series of antioxidant programs (17). Also, garlic and garlic extract has shown to facilitate Nrf2-HO-1 signaling in endothelial (18) and B35 neural cells (19). Nuclear factor (erythroid-derived 2)-like 2, also known as Nrf2, is a transcription factor that protects against oxidative damage triggered by injury and inflammation. Nrf2 is a basic leucine zipper (bZIP) protein that regulates a variety of detoxification enzymes such as heme oxygenase-1 (HO-1), NAD(P)H: quinone oxidoreductase 1 (NQO1). In many cancer cell lines and tumor tissues, high level of Nrf2 is detected, and Nrf2 is thought to play dual roles in cancer cell growth and survival (20).

In this study, we used both rapamycin and SAMC as anticancer reagents in human colon cancer cells and tumor xenograft mice, and investigated the underlying mechanisms of tumor growth inhibition, especially the relationship between autophagy and Nrf2.

## Materials and methods

**Reagents.** SAMC (purity of 99%) was synthesized and purified in our laboratory with a modified procedure as previously reported (21). SAMC was freshly prepared as a stock solution in PBS for the *in vitro* assay and was suspended in 10% (w/v) L-dextrose, 1% (w/v) gum Arabic (Sigma-Aldrich, St. Louis, MO, USA) for application in mice.

**Cell culture.** The human colorectal cancer cell line HCT-116 was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). HCT-116 cells were cultured in RPMI-1640 (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (BI, Cromwell, CT, USA), 100 U/ml of penicillin and 100 mg/ml streptomycin (Solarbio, Beijing, China). All cells were maintained in a humidified incubator at 37°C in 5% CO<sub>2</sub>/95% air.

**Cell viability assay.** A stock solution of SAMC (5 mM) was prepared fresh in PBS. Rapamycin was dissolved in dimethyl sulfoxide (DMSO) as a 5-mM stock solution and dilutions were made in RPMI-1640. The total DMSO concentrations were kept below 0.05% (v/v), which showed no influence on cell growth. Cells were seeded in 96-well plates at a concentration of  $1.5 \times 10^3$  cells/well. After 24 h, cells were categorized into five groups: control, rapamycin (Rapa) (0.5 μM), SAMC (200 and 400 μM) and Rapa+SAMC combination group. The two doses of SAMC were selected based on our previous experiment (data not shown). Cell viability was measured by the SRB method. Briefly, the treated cells were then fixed with 10% TCA for 1 h at 4°C, the 96-well plates were washed three times with distilled water and allowed to dry in the air. Each well was added with 100 μl of sulphorhodamine (SRB) solution and the staining was completed at room temperature for 15 min. The SRB stain solution was removed by washing the plates quickly

with 1% (v/v) acetic acid three times, and the plates were dried in the air. The dried materials in each well were solubilized by adding 200 μl of 10 mM unbuffered Tris base (pH 10.5). The cell viability was detected by measuring the absorbance at 540 nm on a plate reader (Safire2, Tecan, France). All experiments were repeated at least three times.

**DAPI staining.** The HCT-116 cells were seeded into 24-well plates for 24 h. Then Rapa and SAMC (200 and 400 μM) were directly added to the well and incubated for 48 h. The treated cells were washed with PBS and fixed with cold methanol/acetone (1:1, stored at -20°C) for 5 min at room temperature. The solution was removed and washed with PBS, and then incubated with the DAPI solution for 10 min at room temperature. Fluorescent cells were observed under a fluorescence microscope (Olympus, Tokyo, Japan).

**Animal experiments.** Female BALB/c nude mice (16 g, aged 5-6 weeks) were purchased from Institute of Laboratory Animal Sciences, Cams&Pumc (Beijing, China, SCXK 2014-0004). Mice were housed under standard conditions (12:12 h light/dark cycle at 25±2°C and 40-70% humidity) in specific pathogen-free (SPF) conditions, with *ad libitum* access to food and water. The protocol of the animal experiments were performed in accordance with the institutional guidelines of the Animal Care and Use Committee of Shandong University.

**In vivo xenograft implantation and tumor growth.** The human colorectal adenocarcinoma HCT-116 cells used for implantation were harvested during log phase growth and resuspended in phosphate-buffered saline (PBS) at  $5 \times 10^7$  cells/ml. Each Balb/c nu/nu mouse was injected s.c. in the right flank with  $1 \times 10^7$  cells (0.2 ml cell suspension). When tumor volume reached ~100 mm<sup>3</sup>, mice were randomly divided into four groups (n=6): the control, rapamycin (Rapa) (5 mg/kg/per two days), S-allylmercaptocysteine (SAMC) (300 mg/kg/d), and Rapa + SAMC combination. All groups were treated for 28 days, Rapa was given as intraperitoneal injection three times a week, and SAMC was given by oral gavage daily.

Mice were carefully observed daily, and tumor volumes and body weights were measured every four days. The diameters of the tumors were measured with a caliper, and the tumor volume was calculated using the formula:  $V = 1/2 \times L \times W^2$ , where length (L) and width (W) were determined in millimeters (mm). The inhibition rate (%) of tumor growth was defined as the ratio of tumor weight to that of the control.

At the end of the experiment, all animals were euthanized. The tumor weight and spleen, liver, kidney weight were measured. Tumors were resected and snap-frozen in liquid nitrogen or transferred at -80°C for western blotting and PCR.

**Western blot analysis.** Tumor tissues were homogenized with RIPA lysis buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS] containing protease and phosphatase inhibitor cocktails (Roche), at 4°C with vortexing. The total protein concentration was determined by the BCA protein assay reagent (Pierce Biomedical Co., Rockford, IL, USA). Tissue lysates were separated using 12% SDS-PAGE and electro-transferred onto the polyvinylidene difluoride (PVDF) membrane. The

Table I. Effect of rapamycin and SAMC on the tumor growth of HCT-116 xenografts in mice.

Group	Tumor volume (mm <sup>3</sup> )		Average tumor weight (g)	Inhibition rate (%)
	Beginning	At the end		
Control	130.93±25.34	1,242.72±311.60	2.42±0.59	
Rapa	135.22±22.24	390.97±62.71 <sup>a</sup>	0.88±0.17 <sup>a</sup>	63.64
SAMC	110.47±17.64	603.42±157.42 <sup>a,b</sup>	0.99±0.14 <sup>a</sup>	59.09
Rapa+SAMC	129.79±20.92	307.84±53.36 <sup>a,b</sup>	0.48±0.11 <sup>a,b</sup>	80.17

Mean ± SD, n=6. <sup>a</sup>p<0.05 vs control. <sup>b</sup>p<0.05 vs Rapa.

protein expression levels were determined using primary antibodies with the appropriate dilution. The PVDF membranes were washed in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBST) and incubated with appropriate secondary antibodies. The immunoreactive bands were visualized by an enhanced chemiluminescence reagent (Millipore) using Alphamager HP system (Cell Biosciences, USA).

The density of each band was measured using Image-Pro Plus, standardized by the density of  $\beta$ -actin. The primary antibodies included those of anti-LC3 (L7543, Sigma-Aldrich), anti-p62 (ab91526, Abcam), anti-Bcl-2 (ab32124, Abcam), anti-Bax (ab32503, Abcam), anti-Nrf2 (C-20) (sc-722, Santa Cruz Biotechnology), anti-NQO1 (ab34173, Abcam), anti-Akt (sc-8312, Santa Cruz Biotechnology), anti-phospho-Akt/Sre-473 (sc-135651, Santa Cruz Biotechnology), p53(ab28, Abcam) and  $\beta$ -actin (TA-09, ZSGB-BIO).

**Quantitative real-time (q-PCR).** Total RNA from the tumor was extracted using TRIzol Reagent (Invitrogen Corp.). Reverse transcription reactions (SuperScript III First-Strand Synthesis system for RT-PCR; Invitrogen Corp., Carlsbad, CA, USA) were performed with 0.5  $\mu$ g of DNase I (Qiagen)-treated RNA. PCR and quantitative real-time PCR (q-PCR) were carried out using the CFX96 Touch™ Real-Time PCR Detection system (Bio-Rad, Laboratories, Inc.). Expression levels of target genes were normalized by concurrent measurement of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels. Primers for target gene amplification were as follows: Nrf2 (NM\_010902.3): 5'-ATGATGGACTTGGA GTTGCC-3', 5'-TCCTGTTCTTCTGGAGTTG-3'; NQO1 (NM\_008706.5): 5'-CGGTATTACGATCCTCCCTCAACA-3, 5'-AGCCTCTACAGCAGCCTCCTTCAT-3'; GAPDH (NM\_008084.2): 5'-ATGTTCCAGTATGACTCCACTCACG-3', 5'-GAAGACACCAGTAGACTCCACGACA-3'.

**Measurements of the oxidative stress markers and antioxidant enzyme activities.** A 10% liver tissue homogenate was prepared with the phosphate buffer saline (50 mM, pH 7.4) using Teflon homogenizer. A part of the homogenate was mixed with an equal volume of 10% trichloroacetic acid (TCA) and centrifuged at 3,000 rpm for 15 min at 4°C. The supernatant was used to determine the content of glutathione (GSH-Px) and malondialdehyde (MDA) (oxidative stress marker) enzymes. The remaining part of the homogenate was centrifuged at 12,000 g for 45 min at 4°C and the supernatant was used for estimation of antioxidant enzymes activities as superoxide

dismutase (SOD) and catalase (CAT) enzymes. All treatments were conducted in an ice bath. The activities of GSH-Px, MDA, SOD and CAT were then assayed using commercial reagent kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

**Statistical analysis.** Data are shown as mean  $\pm$  SD. Statistical differences were analyzed by one-way ANOVA followed by Bonferroni test for multiple comparisons using GraphPad Prism software. Differences were considered significant at p<0.05. Statistical analysis was performed with SPSS/Win 13.0 software (SPSS, Inc., Chicago, IL, USA).

## Results

**Rapamycin and SAMC combination inhibits colon cancer cell proliferation and induces apoptosis in HCT-116 cells.** To assess the antiproliferative activity of rapamycin, SAMC and co-treatment group, HCT-116 cells were exposed to different concentrations (200 and 400  $\mu$ M) of SAMC for 48 h and assayed for proliferation using the SRB method. As shown in Fig. 1A, the proliferation of HCT-116 cells was significantly suppressed by Rapa, SAMC, and the combination.

SAMC has been shown to induce colorectal carcinoma cell apoptosis (22). To detect the cell apoptosis induced by rapamycin and SAMC, the HCT-116 cells were stained with DAPI to assess the morphological change in this study. The cells exhibited typical morphological signs of apoptosis, such as fragmented nuclei and apoptotic bodies, as evidenced by the arrows in Fig. 1B.

**SAMC potentiates antitumor effect of rapamycin in mouse subcutaneous HCT-116 xenograft tumor models.** The antitumor effect of rapamycin and SAMC was evaluated in the mouse model of HCT-116 xenografts. Compared with the control group, Rapa treatment (10 mg/kg per day three times a week) significantly inhibited tumor growth (Fig. 2A and B) by 63.64% (p<0.05), while SAMC alone inhibited tumor growth by 59.09% (p<0.05), respectively. However, the tumor growth inhibition rate of the combination reached 80.17% (p<0.05) compared with the control. The average tumor volumes on day 28 were 390.97±62.71, 603.42±157.42 and 307.84±53.36 mm<sup>3</sup> in Rapa, SAMC and combination groups, respectively, compared to 1,242.72±311.60 mm<sup>3</sup> of control group (Table I). The body weight of mice decreased in the control group, and Rapa treatment showed a similar

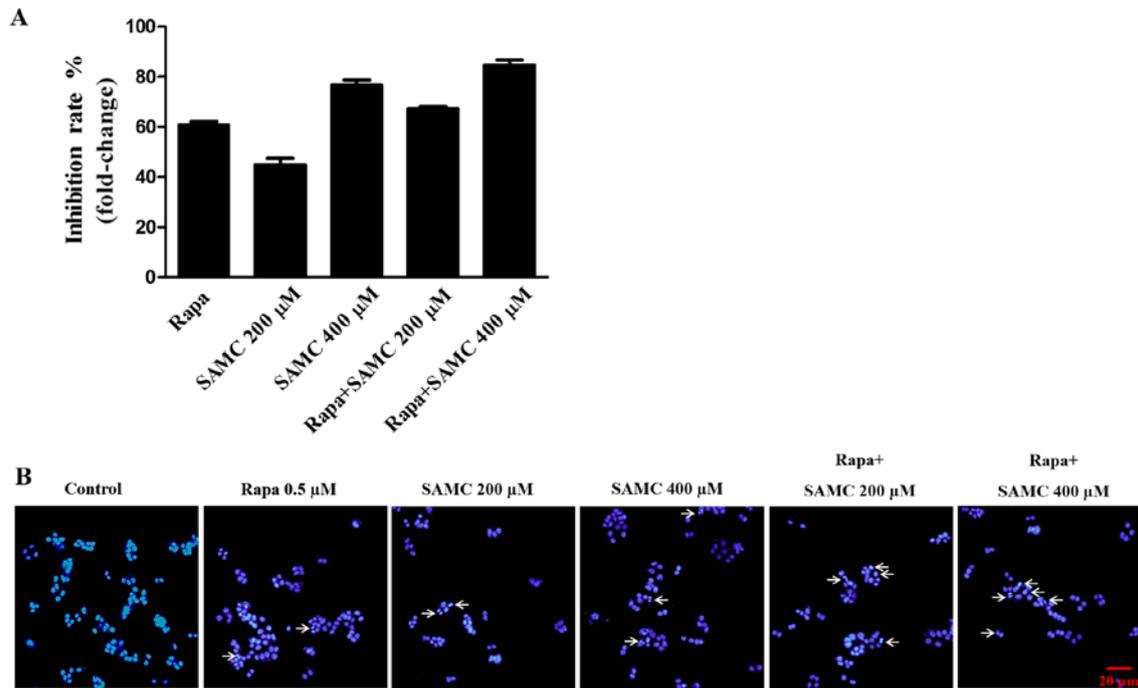


Figure 1. Rapamycin and SAMC inhibit HCT-116 cell growth and induce cell apoptosis. (A) The inhibition effect of Rapa (0.5  $\mu$ M) and SAMC (200 and 400  $\mu$ M) on HCT-116 cell growth was estimated by the SRB assay. Cells were exposed to Rapa and SAMC for 48 h, followed by SRB assay. (B) Rapa and SAMC induced apoptosis showed by DAPI staining of HCT-116 cells after 48-h treatment. Data represent the mean  $\pm$  SD of three independent experiments.

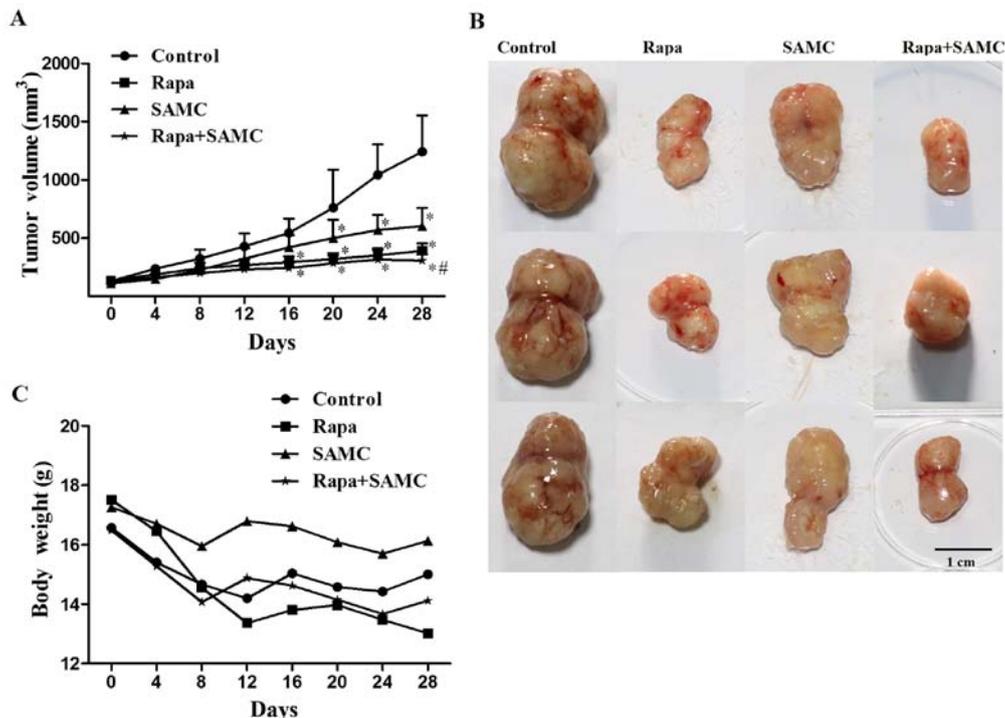


Figure 2. The combination of rapamycin and SAMC inhibit tumor growth in nude mice bearing HCT-116 xenograft. (A) Mice were administered Rapa (5 mg/kg, i.p., three times per week) and SAMC (300 mg/kg, o.p., daily) for 28 days, and tumor volume were measured every other four days (n=6). At the end of treatment, Rapa and SAMC exhibited significant tumor growth inhibition effect, and this effect was strengthened when combination use. Bars, SD. (B) Representative images of tumor tissues in each group. (C) The body weight change of mice in each group. \* $p$ <0.05 vs control; # $p$ <0.05 vs Rapa.

degree of body weight loss to the control; but SAMC treatment showed no obvious weight loss. Moreover, SAMC and Rapa combination partially reversed the weight loss caused by Rapa (Fig. 2C).

*Rapamycin and SAMC combination regulates autophagy process and induces apoptosis.* In order to examine whether autophagy inhibition can enhance the antitumor ability of rapamycin on colorectal cancer, we tested the tumor tissues of

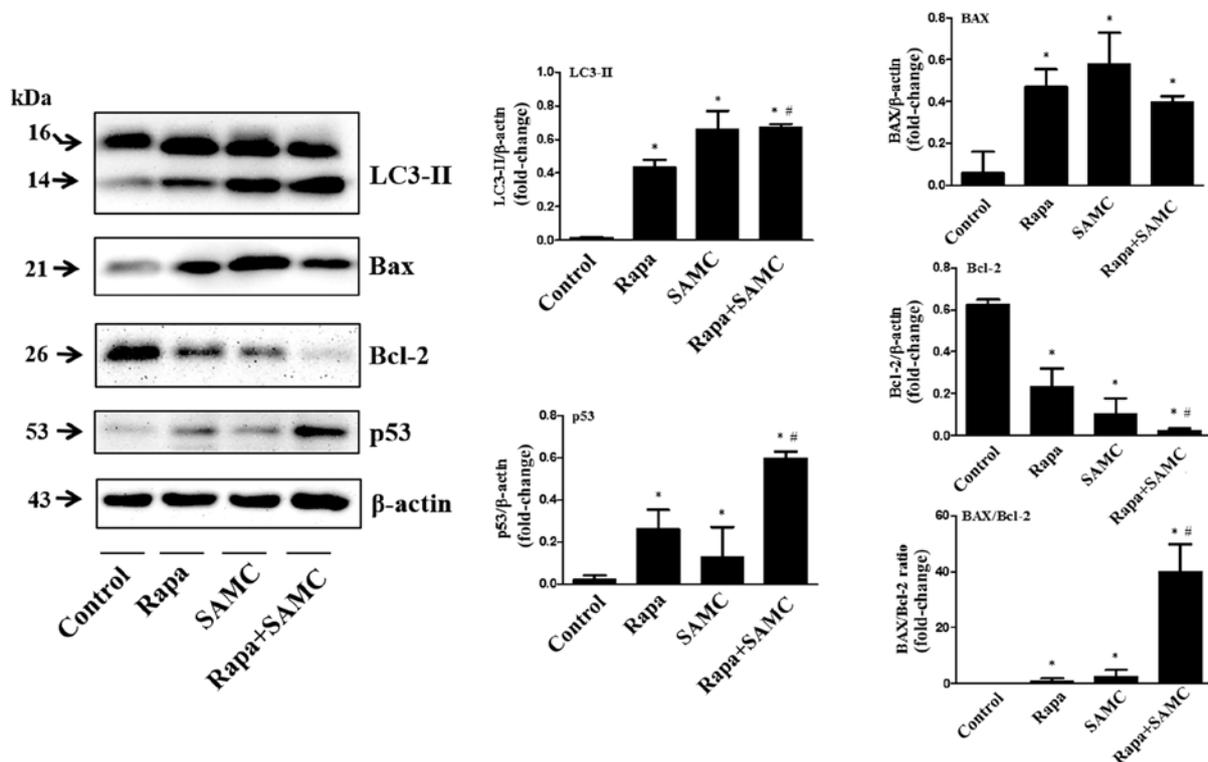


Figure 3. The effect of rapamycin and SAMC treatment on autophagy and apoptosis. The xenografts tumor tissue were examined by western blotting (n=4 in each group). Protein expression levels were normalized to actin. The autophagy marker LC3-II was upregulated by Rapa treatment and enhanced in Rapa and SAMC combination group. The Rapa and SAMC treatment can induce tumor cell apoptosis, which was p53-dependent, as the increased Bax/Bcl-2 ratio and p53 expression. Data represent the mean  $\pm$  SD of three independent experiments (bars, SD). \*p<0.05 vs control; #p<0.05 vs Rapa.

HCT-116 xenografts treated with both Rapa and SAMC. The autophagy-related protein, LC3-II, represent the autophagic activity (23). Western blot analysis showed that the LC3-II expression level was increased by Rapa alone and further increased when combined with SAMC (Fig. 3).

Apoptotic protein Bax and Bcl-2 expression levels were examined in Rapa with/without SAMC-treated tumors. Rapa and SAMC increased the Bax expression, while down-regulated the Bcl-2 expression. The Bax/Bcl-2 ratio increased ~40-fold in the Rapa and SAMC combination group compared to the control (Fig. 3). Tumor suppressor p53 plays a critical role in the induction of apoptosis, which was also examined by western blotting in this study. Rapa increased p53 expression, and further enhanced in the combination group (Fig. 3), indicating that the apoptosis may be p53-dependent.

*Rapamycin and SAMC combination reverses rapamycin-induced upregulation of Akt signaling.* The serine/threonine kinase mTOR is a downstream effector of the PI3K/AKT pathway, and mTOR inhibition consequently reactivates Akt signaling (24). The Akt signaling activation plays a role of drug-resistance in rapamycin-related cancer therapy, so we examined the Akt phosphorylation in tumor tissues. Rapa treatment alone increased Akt phosphorylation, but SAMC alone did not change the Akt status. However, the Akt activation was inhibited in the combination group (Fig. 4).

*Co-treatment of rapamycin and SAMC suppresses tumor growth by activating antioxidant system via regulating p62.* To further explore the mechanism of tumor-suppressing effects

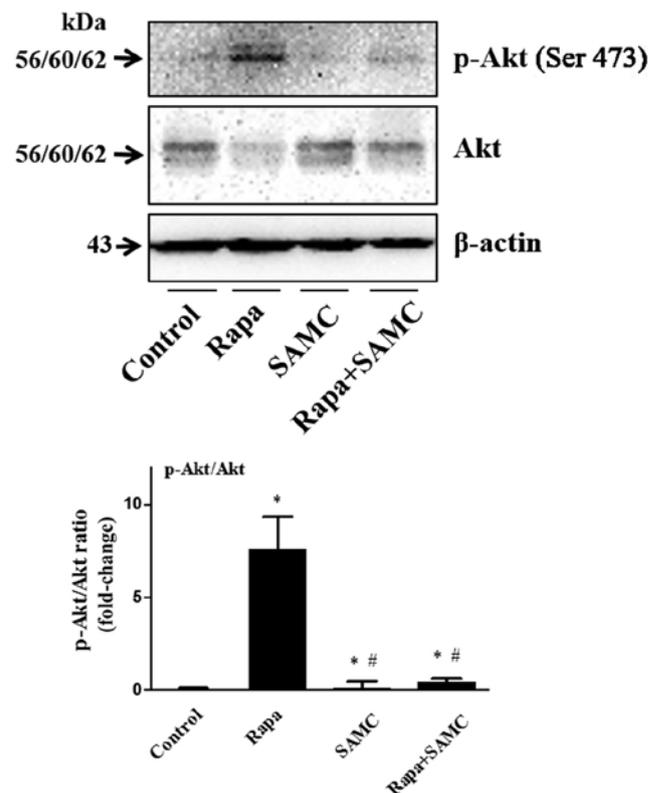


Figure 4. The combination of Rapa with SAMC decreased rapamycin-induced Akt activation. Western blotting results showed that Rapa treatment increased Akt phosphorylation (p-Akt) expression, but was reversed by SAMC combination treatment. Data represent the mean  $\pm$  SD of three independent experiments (bars, SD). \*p<0.05 vs control; #p<0.05 vs Rapa.

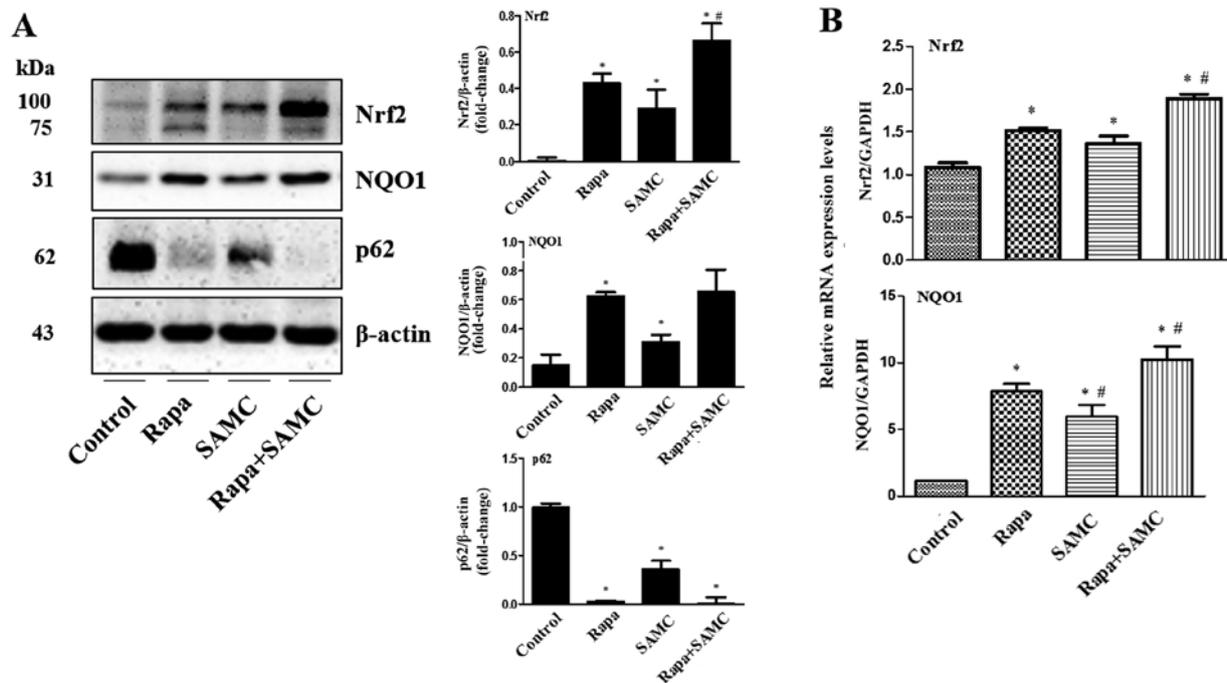


Figure 5. Rapamycin and SAMC activate the antioxidant system in tumor tissue. The antioxidative stress transcription factor Nrf2 and its downstream gene NQO1 expression were increased by western blot analysis (A) and q-PCR (B) in Rapa and SAMC co-treatment ( $n=4$  in each group). The p62 expression was also examined by western blotting (A), which showed a negative expression compare with Nrf2 and NQO1. Data represent the mean  $\pm$  SD of three independent experiments (bars, SD). \* $p<0.05$  vs control; # $p<0.05$  vs Rapa.

Table II. SOD, GSH-Px, CAT activity and MDA level in HCT-116 xenograft mouse liver tissue.

Group	SOD (U/mg protein)	GSH-Px (U/mg protein)	CAT (U/mg protein)	MDA (nmol/mg protein)
Control	167.42 $\pm$ 11.26	103.40 $\pm$ 12.33	11.08 $\pm$ 0.33	5.76 $\pm$ 0.18
Rapa	241.04 $\pm$ 12.27 <sup>a</sup>	164.21 $\pm$ 10.38 <sup>a</sup>	16.20 $\pm$ 0.38 <sup>a</sup>	3.05 $\pm$ 0.06 <sup>a</sup>
SAMC	216.44 $\pm$ 9.43 <sup>a</sup>	136.74 $\pm$ 20.05	13.74 $\pm$ 0.05 <sup>a</sup>	3.28 $\pm$ 0.13 <sup>a</sup>
Rapa+SAMC	287.19 $\pm$ 4.53 <sup>a,b</sup>	183.74 $\pm$ 22.05 <sup>a</sup>	18.30 $\pm$ 2.67 <sup>a</sup>	2.81 $\pm$ 0.04 <sup>a,b</sup>

Mean  $\pm$  SD,  $n=6$ . <sup>a</sup> $p<0.05$  vs control. <sup>b</sup> $p<0.05$  vs Rapa.

by the combination of rapamycin and SAMC, we examined the antioxidant transcription factor Nrf2 and its downstream gene expression such as NQO1. In Fig. 5, western blotting and q-PCR results show that the Nrf2 expression was elevated in the Rapa-treated group compared to the control, and further enhanced when combined with SAMC. While the downstream gene NQO1 expression was consistent with Nrf2 expression.

Autophagy has been shown to suppress tumorigenesis and the key associate point relied on p62/SQSTM1, promoting oxidative stress status (25). Our results indicate that the Rapa, SAMC and combination groups downregulated p62 level compared to the control (Fig. 5A).

*Effects of rapamycin and SAMC on SOD, GSH-Px, CAT and MDA.* To further explore the mechanisms of rapamycin, SAMC and combination for tumor growth inhibition, we determined the activities of antioxidant enzyme SOD, GSH-Px and CAT as well as the lipid peroxidation marker MDA content in

mouse liver tissues. The Rapa alone and combination treatment decreased oxidative stress as indicated by enhanced SOD, GSH-Px and CAT contents and diminishment of MDA level in liver tissues (Table II). The levels of SOD, GSH-Px and CAT were increased by 43.97, 58.81 and 11.08%, respectively, and the MDA was decreased by 47.05% in the Rapa-treated mice compared to the control group. The antioxidant effect of the combination treatment was further strengthened, the SOD, GSH-Px and CAT values were 287.19 $\pm$ 4.53, 183.74 $\pm$ 22.05 and 18.30 $\pm$ 2.67 (U/mg protein), respectively, and MDA content was diminished to 2.81 $\pm$ 0.04 (nmol/mg protein), which were significantly different from the control group.

## Discussion

In cancer therapy, the combination therapeutic regimen regulating autophagic pathway has important therapeutic effects (26). Mechanistic target of rapamycin (mTOR) is an

essential mediator in tumorigenesis, and rapamycin has been shown to induce cancer cell death by stimulating autophagy or apoptosis (9). Autophagy inhibition has been examined by many research laboratories, indicating that it may be a novel way of increasing the efficacy of anticancer agents (27).

Garlic has been used in traditional Chinese medicine (TCM) for centuries for its anti-inflammatory, cardiovascular protection, and anticancer effects (28). SAMC, a water-soluble garlic derivative, has been shown to have antiproliferation ability in many cancer cell lines, and tumor suppressing effect under *in vivo* conditions (29). Thus, we explored the anticancer effects of SAMC and combination use with rapamycin as well as their molecular mechanisms.

In this study, we showed that rapamycin suppressed tumor growth in the HCT-116 xenograft mouse model, and this effect was enhanced when combined with SAMC. In brief, the SAMC treatment upregulated the expression level of Bax, whereas rapamycin downregulated Bcl-2. As a consequence, the combination of rapamycin and SAMC markedly raised the Bax/Bcl-2 ratio and significantly increased the induction of apoptosis in colorectal cancer. The p53-dependent apoptosis observed was in agreement with published results that the activation of p53 mediates the upregulation of Bax and downregulation of Bcl-2 (30).

In cancer cells and its microenvironment, hypoxia and nutrient-deprivation condition could induce the autophagy process. Autophagy breaks down cellular damaged organelles and accumulates proteins for recycling, and the catabolites are recycled and used for biosynthesis and energy-metabolism as a cytoprotective response, which is essential for cancer cell survival (31). SAMC has been demonstrated to enhance autophagy in a liver disease model (32). We tested the autophagy activity in colorectal cancer tumor tissues. Western blotting results show that rapamycin and SAMC treatments increased the autophagic marker LC3-II protein expression. These results demonstrate that co-treatment of rapamycin and SAMC in colorectal cancer tumors can activate the autophagy pathway.

Because mTOR signaling contributes to drug resistance in patients (33), and one suggestion is that there is a mTOR-mediated negative feedback loop to Akt (34), we tested the Akt phosphorylation in colorectal cancer. The phosphorylation of Akt (p-Akt) expression was elevated in the rapamycin treatment group, which was reversed with the combination treatment. These results demonstrate that SAMC could be a good adjuvant with rapamycin in cancer treatment.

The hypoxia condition in tumors increases oxidative stress, which will activate the master regulator of antioxidant defense regulator Nrf2 which participates in tumor growth (35,36). Garlic and DATS has been shown to participate in Nrf2-antioxidant responses (37). In our study, western blotting and q-PCR analyses both confirmed that rapamycin increased Nrf2 expression and the downstream gene NQO-1, the antioxidant protein expression was further enhanced with the combination treatment, indicating that rapamycin and SAMC could suppress tumor growth by the Nrf2 signaling pathway. Since the interaction between Nrf2 and autophagy plays a key role in tumorigenesis (38), we explored the autophagosome cargo protein p62/SQSTM1 expression. Previous study has demonstrated that autophagy can suppress tumorigenesis through

elimination of p62. Our results indicated that p62 in tumor was downregulated by the rapamycin treatment with/without SAMC, which was consistent with the recent report that Nrf2-Keap1 binding competed p62 (39) for autophagy degradation (40). So, the Nrf2-Keap1 system activated by rapamycin and SAMC co-treatment in HCT-116 xenograft mice could be related to autophagy through p62, which is a key pathway in tumorigenesis and cancer therapy.

It is well-known that levels of SOD, GSH-Px, CAT and MDA are considered as common indexes of tissue antioxidant status (41). The MDA level is widely used as a marker of lipid peroxidation damage. Furthermore, inhibition of antioxidant SOD, MDA and GSH enzymes is implicated in the pathogenesis conditions. SAMC has been reported to reduce oxidative stress and inflammation in liver-injury mice (42). Our study results show that rapamycin with/without SAMC treatment effectively increased the liver antioxidative capacity and alleviated the oxidative stress conditions.

In conclusion, these results showed that the rapamycin and SAMC combination induced apoptosis, activation of autophagy, and downregulation of p62. Additionally, this combination reversed the oxidative stress condition by activating antioxidative transcription Nrf2 and downstream gene NQO1 as well as increased SOD, GSH-Px, and CAT activities and decreased the MDA level in liver tissues. Therefore, combining rapamycin and SAMC for the treatment of colorectal cancer might be feasible in clinical use. The underlying mechanism of autophagy/p62/Nrf2 pathway revealed in this study may provide a new direction for drug development.

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