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# Anisomycin represses in vitro and in vivo colon adenocarcinoma CT26 cell growth via activation of caspase-cascade with reduction of carcinoembryonic antigen

### Pengtao You, Haifeng Fu, Zhiwei Zhou, Yuan Wang, Manman Sun, Jing Liu, Feiyue Xing

## ABSTRACT

**Aims:** In vitro evidence indicates that anisomycin can induce cell apoptosis. This study was to evaluate potential of anisomycin to treat colon adenocarcinoma in vitro and in vivo. **Methods:** Cell viability was determined by methyl thiazolyl-tetrazolium bromide (MTT). Cell cycle and apoptosis were detected by flow cytometry. CT26 colon adenocarcinoma model was established. Cellular apoptosis and tumor necrosis were detected by TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling), and H&E staining. Carcinoembryonic antigen (CEA) was detected by in situ immunofluorescence. Expressions of caspases were measured by Western Blot. **Results:** Our results showed that multipoint intratumoral administration of anisomycin significantly suppressed colon adenocarcinoma CT26 cell growth, and resulted in the survival of approximately 80% of CT26-bearing mice 50 days after CT26 inoculation, superior dramatically to adriamycin. Anisomycin inhibited the proliferation of CT26 cells, and arrested the cells into S and G2/M phases with the production of sub-diploid cells. Anisomycin induced in vitro and in vivo apoptosis of CT26 cells, which was consistent with the enhanced expressions of caspases. Specially, the apoptotic rate of the tumor cells in the anisomycin-treated mice was higher than that in the adriamycin-treated mice, which was supported by the observed histopathological and immunochemical changes in the tumor tissue. Carcinoembryonic antigen-positive cells in the tumor tissues also were prominently decreased by anisomycin. **Conclusion:** These results indicate first time that anisomycin efficaciously represses growth of colon adenocarcinoma with the extended survival through activation of caspase signaling with reduction of CEA, significantly superior to adriamycin. Thus, it is a promising drug to be applied to colon adenocarcinoma therapy.

**Keywords: Anisomycin, Apoptosis, Carcinoembryonic antigen, Colon adenocarcinoma**

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## INTRODUCTION

Anisomycin (2-(p-methoxybenzyl)-3,4-pyrrolidine-diol-3-acetate) is a pyrrolidine antibiotic, produced by *Streptomyces griseolus* [1], which inhibits translation by binding to the 60S ribosomal subunits in many types of cells, blocking the peptide bond formation and suppressing the peptidyltransferase reaction, thereby preventing elongation and causing polysome stabilization [2, 3]. Expressions of genes related to protein synthesis, such as eukaryotic translation initiation factor 4 family proteins and ribosomal proteins, could be inhibited by anisomycin [4]. Currently, anisomycin has been used in memory research, showing that it is intimately implicated in learning and memory, traumatic memory, and recovery of memory [5–8].

On the other hand, it was found that anisomycin induced cell apoptosis [9–11]. This role is typically related to activation of p38 mitogen activated protein kinase (MAPK) [12]. During the process of apoptosis, the apoptosis-promoting proteins are up-regulated and cytochrome c is released from the mitochondria in leukemia cells [13, 14]. Hori et al. studies also showed mitochondrial membrane potential collapse and cytochrome c release into the cytosol in anisomycin-treated U937 cells [4]. Our previous studies showed that anisomycin could inhibit in vitro proliferation of differently histological types of tumor cells [15]. Based on this, we applied first anisomycin to therapy of Ehrlich ascites carcinoma (EAC)-bearing mice, and found that it efficaciously extended the mouse survival [16]. Whether does in vivo therapeutic effect of anisomycin exist in other histological types of tumors? Due to high morbility and mortality of digestive system tumors in the world, in the current study colon adenocarcinoma was tested first time with anisomycin from in vitro to in vivo.

## MATERIALS AND METHODS

*Animals:* Male BALB/c mice, 6–8 weeks old, 20±2 grams, were supplied by the Guangdong Medical Animal Center (Guangzhou, China). These animals were fed in SPF lab of Experimental Animal Center of Jinan University. All animal handling and experimental procedures were approved by the animal care and use committee of guangdong medical animal center.

*Cell culture and treatment:* CT26 colon adenocarcinoma cell line, donated from Institutes of Life and Engineering, Jinan University, was cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS) (Gibco-BRL, USA) at 37˚C in humidified air with 5% $CO_2$. Cells were incubated for different times with anisomycin (Sigma-Aldrich, St. Louis, MO, USA) at 1, 5, 10, 20, 40, 80 and 160 ng/mL and then subjected to analysis.

*Cell viability:* CT26 cells were incubated in 96-wells plates at the density of $1 \times 10^4$ cells/well/200 μL. The cells were treated or untreated with different concentrations of anisomycin (1, 5, 10, 20, 40, 80, and 160 ng/mL) and 500 ng/mL adriamycin (Zhejiang Hisun Pharmaceutical Co. Ltd., Taizhou, China) for 48 h or 40 ng/mL anisomycin and 500 ng/mL adriamycin for 6, 12, 24, 36, 48, and 72 h. 20 μL of 5 mg/mL of methyl thiazolyl-tetrazolium bromide (MTT) (Gibco-BRL) was added for four hours. Afterwards, the supernatant of each well was replaced by dimethyl sulfoxide (DMSO) to dissolve the formazan product of MTT reduction. Absorbance was measured at 570 nm using a Model 680 microplate reader (Bio-Rad, Inc. USA).

*In vivo therapy:* 100 μL of suspension with CT26 cells ($1 \times 10^7$ cells/mL) was subcutaneously-inoculated into each animal at the right back. Inoculated mice were then divided randomly into three groups (mice/group). The experimental therapy was carried out when the tumor volume reached about 50 mm³. Anisomycin (5 mg/kg), adriamycin (5 mg/kg) or 100 μL of PBS was intratumorally injected into the CT26-bearing mice once a day for 14 times. Solid tumor sizes were measured every day to calculate tumor volume using the following formula:

Tumor volume = (length x width²)/2, where length and width are given in mm.

*Flow cytometry:* The CT26 cells treated above in vitro and separated from in vivo tumor tissue were stained using an Annexin V/FITC kit containing propidium iodide (PI) (KeyGEN Biotech, China), according to the manufacturer's instructions and analyzed using a flow cytometer with a Cell Quest software (FACSCalibur, Becton Dickinson, USA).

*Western Blot:* For the in vitro experiment, CT26 cells were incubated in 12-well plates with different concentrations of anisomycin for 48 h. For the in vivo experiment, tumor tissues of each group were triturated into homogenate. Erythrocyte lysis buffer was added for 5 minutes. A RIPA Lysis kit (Beyotime, China) containing phenylmethanesulfonyl fluoride was used to lyse the cells. An equal amount of the samples was separated by SDS-PAGE and transferred on nitrocellulose membranes (Pall Corporation, USA). The membranes were incubated overnight with anti-caspase-3, anti-cleaved-caspase-3, anti-cleaved-caspase-8 and anti-cleaved-caspase-9 antibodies (1:1,000 dilution) (Cell Signaling Technology, Inc. USA), respectively. Then, horse-radish-peroxidase-conjugated IgG antibody (1:2,000 dilution) (Cell Signaling Technology) was added to incubate the membranes for 1 h. The membranes were finally developed with ECL Western blotting detection reagent (Thermo Fisher Scientific, Rockford, IL, USA). The band density was tested by the FluorChem 8000 system.

*Histopathology:* The tumor tissues from each group were resected and fixed overnight in 4% paraformaldehyde solution. They were then embedded in paraffin and

processed for construction of 4-μm paraffin-embedded sections using a microtome. The paraffin sections were stained with hematoxylin and eosin (H&E) staining, and were then photographed under a light microscope equipped with an OPTEC DV200 digital camera (OPTEC, Ltd., China) to assess infiltration of inflammatory cells in the tumor tissues.

*TUNEL assay:* To evaluate apoptosis in tumor tissue, a terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-digoxigenin nick-end labeling (TUNEL) assay was applied using a TUNEL kit (KeyGEN Biotech, China), following the manufacturer's instructions. The stained tissue sections were then examined and photographed under a light microscope equipped with an OPTEC DV200 digital camera (OPTEC, Ltd., China).

*In situ immunofluorescence staining:* Furthermore, tissue sections were deparaffinized and processed by fluorescence in situ immunofluorescence staining. The sections were incubated with an anti-carcinoembryonic antigen antibody (1:500 dilution) (Abcam, Plc. UK) overnight at 4°C, followed by Alexa Fluor 488-conjugated anti-rabbit antibody (1:1,000 dilution)(Cell Signaling Technology, Inc. USA) at 37°C for 1 h, and examined under a Leica DMRA2 fluorescence microscope with FW 4000 software (both from Leica, Germany).

*Statistical analysis:* Comparison between sets of two groups was performed using the Student's t-test, while sets of more than two groups were compared by ANOVA. Differences between means were considered significant when the $p$-value was less than 0.05.

## RESULTS

Anisomycin inhibits the proliferation of CT26 cells and arrests the cell cycle. Effects of anisomycin on growth of CT26 cells were examined by MTT assay. Compared to the control, anisomycin resulted in a significantly inhibitory effect on the proliferation of CT26 cells in a dose-dependent manner. The cell viability was decreased to 20.59% when the cells were treated with 40 ng/mL anisomycin for 48 h, higher than that in the adriamycin-treated cells (Figure 1A), and increased with the prolonged exposure to anisomycin. 24 hours after the treatment, a dramatic inhibition of cell viability was observed in the anisomycin-treated cells when compared to the cells treated with 500 ng/mL adriamycin (Figure 1B). To examine the role of anisomycin in CT26-cell cycle, CT26 cells were stimulated with the different doses of anisomycin for 48 h and subjected to flow cytometry for cell cycle analysis. Compared to the control, the treatment with anisomycin resulted in decrease of the cells at the G0/G1 phase and increase at the sub-G0/G1 phase with increasing doses of anisomycin (Figure 1C–D). These data
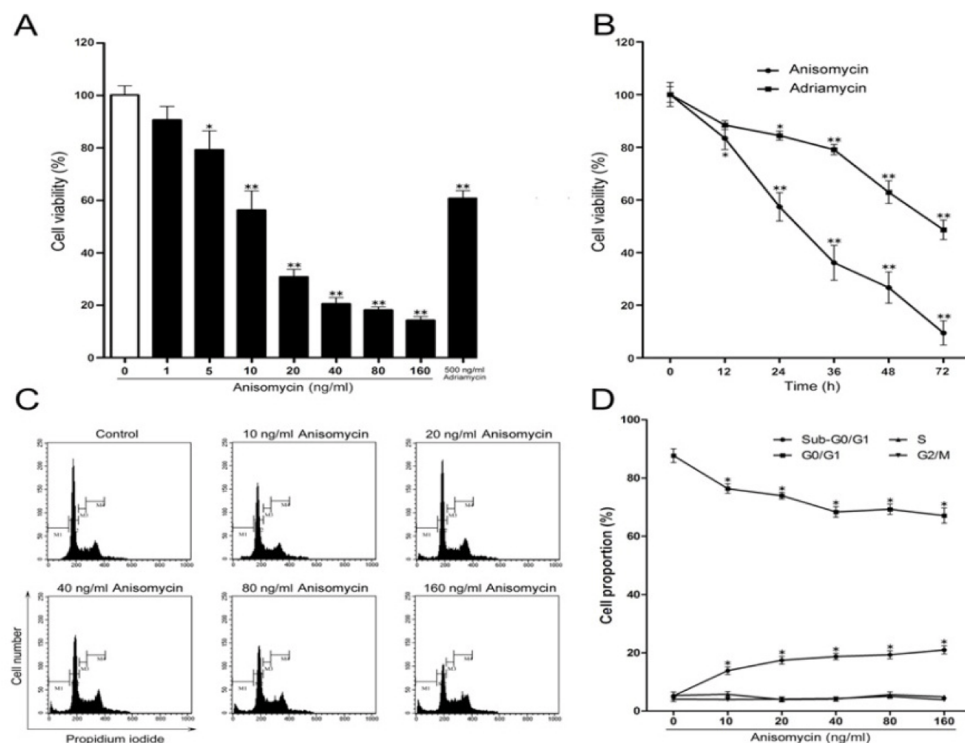


Figure 1: Effect of anisomycin on the in vitro proliferation and cell cycle progression of CT26 cells. (A) Following various treatments with anisomycin or 500 ng/mL adriamycin, the cell viability was determined using the MTT assay, (B) Following treatment with 40 ng/mL anisomycin or 500 ng/mL adriamycin, the cell viability was determined at the indicated time, (C) The cells were fixed with ethanol and stained with propidium iodide, and cell cycle distribution was analyzed by flow cytometry, and (D) The percentage of the cells at G0/G1, S and G2/M phases was shown. All data are represented as the means of 3 independent experiments. *$p < 0.05$ and **$p < 0.01$ vs the control group.

demonstrate that anisomycin markedly inhibits the cell proliferation, arrests the cell cycle and promotes the cell apoptosis, appearing in a dose-dependent relationship.

Anisomycin extends the survival of CT26-bearing mice. The tumor volume of the mice treated with anisomycin or adriamycin displayed almost the same volume, much lower than that of the control. Within 40 days after the inoculation of CT26 cells, the therapeutic effect of anisomycin appeared to be equal to that of adriamycin (Figure 2A–B). The percentage of survival in CT26-bearing mice in the control and 5 mg/kg adriamycin-treated mice was about 40% at 40 d after the inoculation and no animal survived beyond 50 d. But the survival rate of the 5 mg/kg anisomycin-treated mice reached about 80% at the same time, much higher than that of the control mice or 5 mg/kg adriamycin-treated mice (Figure 2C). As shown in Figure 2D, the tumor weights in the anisomycin-treated mice was much lower than that in the control, but there was no statistical difference between the tumor weight of the adriamycin-treated mice and the anisomycin-treated mice. Compared with the control, H&E staining showed that dissimilar to the untreated control, the tumor tissues in the adriamycin-treated or anisomycin-treated mice had big area necrosis. The observed reduction of the inflammatory cell infiltration around the tumor tissues in adriamycin-treated mice might be related to the tumor tissue necrosis (Figure 2E). Although the tumor volume and weight in the adriamycin-treated mice were similar to those in the anisomycin-treated mice, the former survival rate was much lower than the latter. We speculated that this might be related to adverse effects of adriamycin.

Effect of anisomycin on immune-organs in CT26-bearing mice unlike adriamycin. Compared with the adriamycin-treated mice, the increase of three organs-to-body ratios was observed in anisomycin-treated mice, but the liver- and spleen-to-body ratios in the both-treated mice all were lower than those in the untreated control (Figure 3A–B). On the contrary, the thymus-to-body ratio in the anisomycin-treated mice was higher than that in the adriamycin-treated mice or the untreated control (Figure 3C). These data suggest that similarly, both anisomycin and adriamycin all inhibit the hyperplasia of peripheral immune organs stimulated by tumor cells, but that dissimilarly, anisomycin may stimulate the hyperplasia of a central immune organ. Therefore, adverse effects of anisomycin on immune organs might be lower than of adriamycin.

Anisomycin induces the apoptosis of CT26 cells via an increase of caspase activity. In vitro CT26 cells were treated with different doses of anisomycin for 48 h and analyzed by flow cytometry. In comparison with the control, the apoptotic rate of CT26 cells was increased with the increasing doses of anisomycin, in which the early-stage and late-stage apoptotic rates of CT26 cells reached 13.93 and 44.22%, respectively (Figure 4A). We further tested apoptotic changes using ex vivo CT26 cells from the treated or untreated mice with the tumor. Compared with
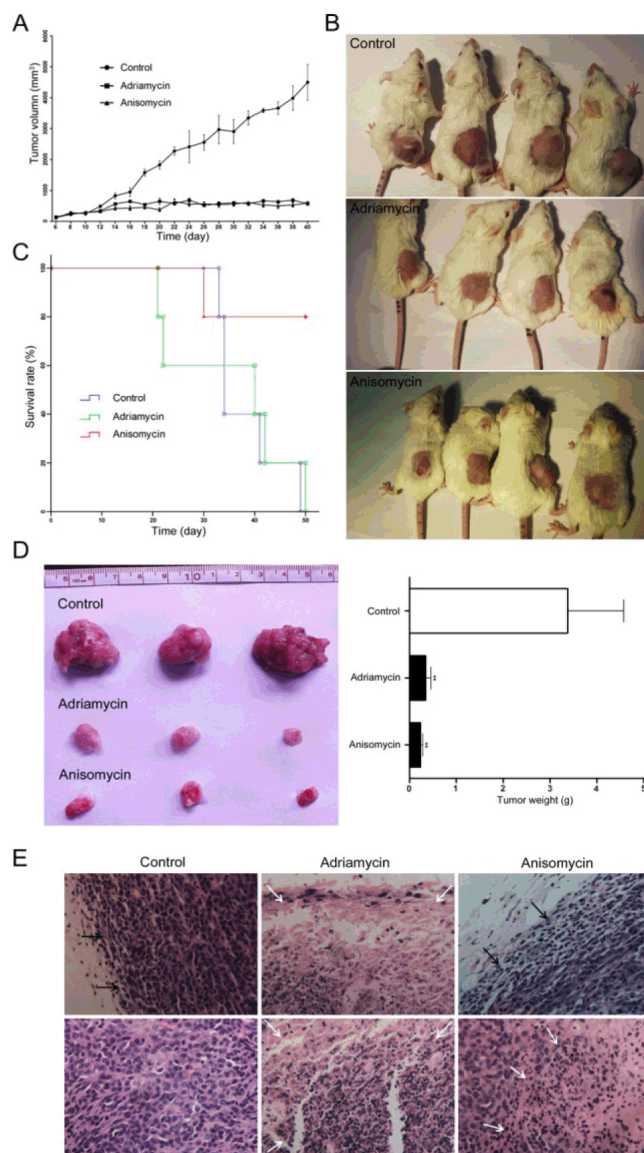


Figure 2: In vivo therapeutic effect of anisomycin on CT26-bearing mice, (A) Tumor volume of the CT26-bearing mice is indicated within 40 days after the treatment with anisomycin or adriamycin, (B) The images of the CT26-bearing mice treated with anisomycin or adriamycin were captured 26 days after cell inoculation, (C) Survival rate of the CT26-bearing mice treated with anisomycin is shown within 50 days after the inoculation of CT26 cells, (D) Images of the tumors and the tumor weight in the mice were presented after the treatment of anisomycin or adriamycin, and (E) Paraffin-embedded tumor tissue sections were stained with H&E. Inflammatory cell infiltration (black arrows) and necrosis (white arrows) in the tumor tissues were observed. *$p < 0.05$ and **$p < 0.01$ vs the control-treated mice.

the control, the apoptotic rate of the tumor cells in the adriamycin-treated and anisomycin-treated mice reached 35.49% and 42.97%, respectively (Figure 4B). Supporting these data, TUNEL assay also showed that there were more in situ apoptotic cells stained in brown and bigger area necrosis of the tumor tissues in the adriamycin- or

anisomycin-treated mice than in the control (Figure 4C). Consistent with the foregoing findings, the expressions of caspase-3, cleaved-caspase-3, cleaved-caspased-8 and cleaved-caspased-9 in the treated CT26 cells were significantly up-regulated with the enhancing doses of anisomycin (Figure 4D). Especially, the four proteins of the tumor tissues in the anisomycin-treated mice were highly expressed more than in the adriamycin-treated mice (Figure 4E). Combining all the in vitro and in vivo results we obtained, it highlights the involvement of the tumor cell apoptosis in the therapeutic effects exerted by anisomycin in vivo.

Anisomycin reduces the expression of CEA on the surface of CT26 cells. CEA is often used as a biomarker of digestive system tumor. Therefore, the level of CEA was determined through in situ immunofluorescence staining. The results showed that compared to the control, the expression of CEA of the tumor tissues in the anisomycin- or adriamycin-treated mice was dramatically reduced, which is mainly distributed on the surface of the tumor cells. Due to the big area necrosis of the tumor tissues in the treated mice, we speculated that the reduction of CEA may be attributed to apoptosis, death and disintegration of a great number of the tumor cells, but not to the decrease of CEA itself (Figure 5).
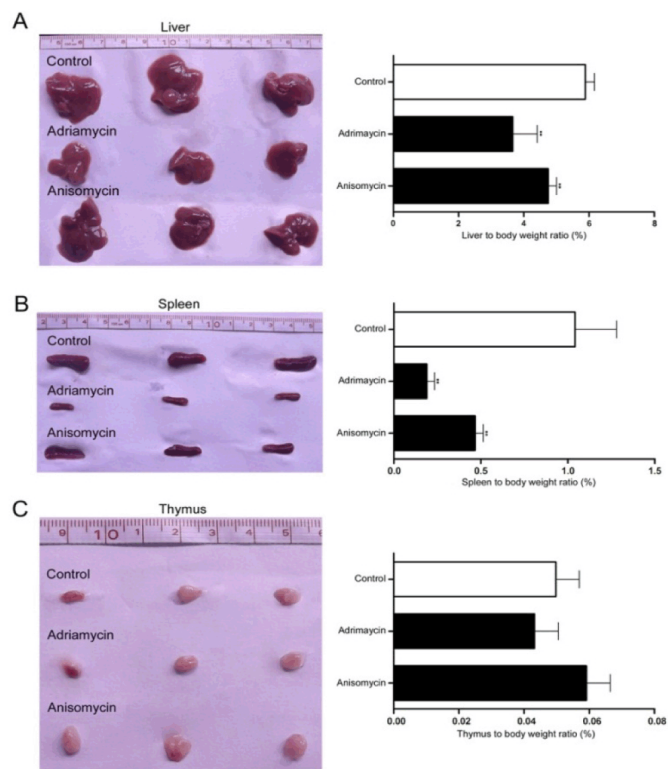


Figure 3: Effects of anisomycin on liver, spleen and thymus in CT26-bearing mice, (A) Images of liver and the liver to body ratio in the CT26-bearing mice treated with anisomycin or adriamycin, (B) Images of spleens and the spleen to body ratio in the CT26-bearing mice treated with anisomycin or adriamycin, and (C) Images of thymus and the thymus to body ratio in the CT26-bearing mice treated with anisomycin or adriamycin. *$p$ < 0.05 and **$p$ < 0.01 vs the control group.
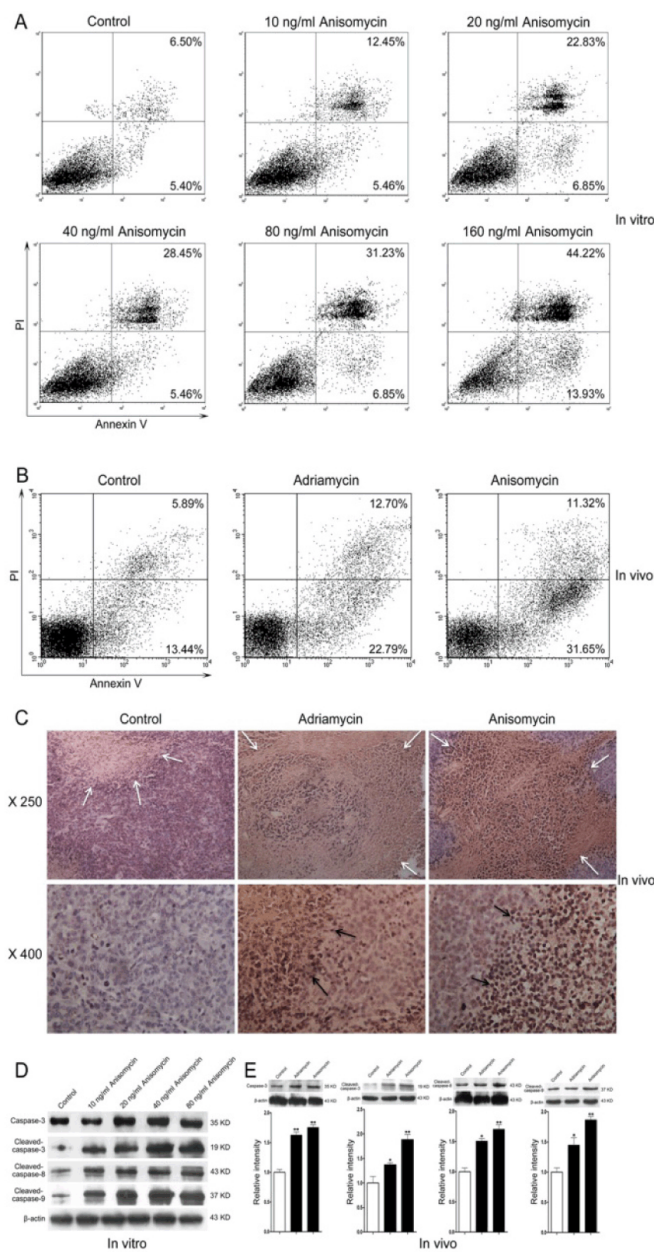


Figure 4: Relationship between caspase-cascade and CT26 cell apoptosis induced by anisomycin, (A) CT26 cells were treated with the different concentrations of anisomycin or 500 ng/mL of adriamycin for 48 h. Then, cell apoptosis was analyzed by flow cytometry, (B) The CT26 cells isolated from the tumor tissues in the CT26-bearing mice treated with anisomycin or Adriamycin once a day for 14 times were analyzed by flow cytometry, (C) Apoptotic cells in the tumor tissues in the CT26-bearing mice treated with anisomycin of adriamycin were detected by TUNEL staining. The black arrows point to apoptotic cells, and the white arrows to necrosis region, (D) The CT26 cells were treated with different concentrations of anisomycin for 48 h in vitro, and levels of caspase proteins were determined by Western blotting, and (E) Levels of caspase proteins in tumor tissues were determined by Western blotting.*$p$ < 0.05 and **$p$ < 0.01 vs the control group.
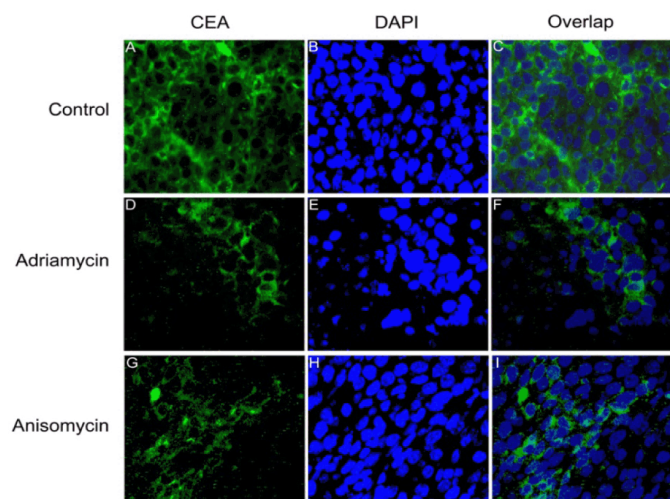
Figure 5: CEA immunofluorescence staining of tumor tissues in CT26-bearing mice treated with anisomycin or adriamycin. Paraffin-embedded tumor tissue sections were stained with polyclonal antibodies specific for CEA (×400, green). The results are representative of at least two independent experiments.

## DISCUSSION

Anisomycin is well known as a protein synthesis inhibitor which binds to the 60S ribosomal subunits and directly inhibits 28S rRNA [17, 18]. It was found that anisomycin efficiently induced cell apoptosis and cell death in vitro. In this study, we first investigated effects of anisomycin on CT26 colon adenocarcinoma in vitro, proving that it efficiently induces CT26 cell apoptosis and death. Recent reports have shown that anisomycin can also enhance death receptor-mediated apoptosis in glioblastoma cells, prostate cancer cells, malignant mesothelioma cells, human hepatoma cells and melanoma cells in vitro [19–23]. Anisomycin induced mouse macrophage apoptosis via p38 pathway, whereas not JNK or ERK1/2 pathway [24]. In ovarian cancer, it promoted the expression of annexin-V mRNA via ERK1/2 but not p38 pathway [25]. Caspase-8 and caspase-8 inhibitor FLIP also have been reported to participate in anisomycin-induced apoptosis [26]. These reports suggest that the apoptotic pathway activated by anisomycin may depend on cell histological types. Our results demonstrated that following the treatment of colon adenocarcinoma by anisomycin the expressions of caspase-3, cleaved-caspase-3, cleaved-caspase-8 and cleaved-caspase-9 were up-regulated by anisomycin in a dose-dependent manner. This spurred us to further test its in vivo therapeutic effect on colon adenocarcinoma.

Since anisomycin is of strongly in vitro induction of tumor cell apoptosis, can we get the same effect on the tumors in vivo? To date, only Liu et al. preliminarily treated TSA (a mouse mammary tumor cell line)-bearing mice intraperitoneally with anisomycin at a dose of 0.5 mg/ mouse. Their data revealed that a low dose of anisomycin was markedly effective in treating TSA-bearing mice [27]. Our previous study demonstrated that anisomycin efficaciously inhibited Ehrlich ascites carcinoma growth and extended the survival of the tumor-bearing mice [16]. In the above groups, they all tested breast cancer. How is an in vivo effect of anisomycin on other tumors? In this study, it efficaciously inhibited CT26 tumor growth, and decreased the tumor volume and weight with the significantly extended survival of CT26-bearing mice, superior to the treatment of an equal dose of adriamycin. These data were consistent with the inhibition of CT26 cell proliferation by the in vitro treatment of anisomycin and the apoptosis of CT26 cells by the in vitro and in vivo treatment of anisomycin. Although the inhibitory rate of anisomycin on the tumor growth was equal to that of adriamycin, the survival time of CT26-bearing mice treated by adriamycin was still much shorter than that by anisomycin. We consider that the shorter survival of the adriamycin-treated mice may be related to tumor cell invasion, distant metastasis and toxicity of adriamycin. Mawji et al. found that mice inoculated intravenously with the tumor cells pretreated by anisomycin in vitro also had a markedly decreased tumor count and area within the liver, lung and bone, suggesting that it may have a prophylactic role in tumors [26].

Interestingly, anisomycin may have both antitumor and immunomodulating activities under the experimental conditions. Our data showed that the liver, spleen and thymus to body weight ratios in the adriamycin-treated mice were lower than those in the anisomycin-treated mice, indicating that adriamycin has more adverse effects than anisomycin. This may be another reason why the adriamycin-treated mice had a lower survival rate than the anisomycin-treated mice. Notably, our data showed that the mouse thymus in the anisomycin-treated group was larger than that in the adriamycin-treated or PBS-treated groups, suggesting that anisomycin may stimulate the hyperplasia of immune organs. The mouse spleens in the anisomycin-treated group were much larger than in the adriamycin-treated groups. This role of anisomycin was also observed in our previous study using a consecutive four-week intravenous administration of anisomycin into normal mice [28]. Therefore, the antitumor effect of anisomycin may be relevant to not only tumor cell apoptosis, but also stimulation of immune organs with less adverse effects.

Some studies show that anisomycin induces cancer cell apoptosis, which results from the activation of the caspase cascade [29, 30]. Our results showed that the apoptosis of the in vitro anisomycin-treated CT26 cells was increased, following the elevation of caspase-3, cleaved-caspase-3, cleaved-caspase-8 and cleaved-caspase-9 protein levels. Simultaneously, the apoptosis of the in vivo anisomycin-treated CT26 cells was also increased after the enhanced expressions of caspase-3, cleaved-caspase-3, cleaved-caspase-8 and cleaved-caspase-9 proteins. The levels of

the four proteins significantly up-regulated by anisomycin were higher than those induced by adriamycin. Moreover, these results were further supported by TUNEL assay and CEA assay. Taken together, these data suggest that anisomycin may induce colon adenocarcinoma cell apoptosis by activating the caspase cascade. Another research group demonstrates that anisomycin induces U251 and U87 cell apoptosis by activating p38 MAPK and JNK but inactivating ERK1/2 [31]. Macrophage inhibitory cytokine-1 (MIC-1) is also a critical inducer of apoptosis-related gene products. Ribotoxic anisomycin induced MIC-1 expression via p38-activated transcription factor 3 pathway and subsequent apoptosis while suppressing survival ERK signal in the colon cancer cells [32]. These data may indicate that a cross talk may exist between MAPK and caspase signaling pathways. Therefore, the detailed mechanism remains to be further elucidated.

## CONCLUSION

In summary, our results indicate that anisomycin efficaciously inhibits colon adenocarcinoma growth and extends the survival of the tumor-bearing mice through activation of caspase signaling with reduction of carcinoembryonic antigen, superior significantly to adriamycin. Moreover, the here used dose of anisomycin causes no obvious side effect. Thus, it is a promising drug to be applied to colon adenocarcinoma therapy. Additional studies are essential to elucidate mechanisms involved in their beneficial actions and their fate in human body.

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## Author Contributions

Pengtao You – Acquisition of data, Analysis and interpretation of data, Drafting the article, Final approval of the version to be published

Haifeng Fu – Acquisition of data, Analysis and interpretation of data, Revising it critically for important intellectual content, Final approval of the version to be published

Zhiwei Zhou – Acquisition of data, Revising it critically for important intellectual content, Final approval of the version to be published

Yuan Wang – Acquisition of data, Revising it critically for important intellectual content, Final approval of the version to be published

Manman Sun – Acquisition of data, Revising it critically for important intellectual content, Final approval of the version to be published

Jing Liu – Substantial contributions to conception and design, Analysis and interpretation of data, Revising it critically for important intellectual content, Final approval of the version to be published

Feiyue Xing – Substantial contributions to conception and design, Analysis and interpretation of data, Drafting the article, Revising it critically for important intellectual content, Final approval of the version to be published

## Guarantor
The corresponding author is the guarantor of submission.

## Conflict of Interest
Authors declare no conflict of interest.

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