mTOR Inhibitor PP242 Increases Antitumor Activity of Sulforaphane by Blocking Akt/mTOR Pathway in Esophageal Squamous Cell Carcinoma

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Abstract

This study aims to explore the anti-tumor activity of Sulforaphane (SFN) alone and combined with Akt/mTOR pathway inhibitors as well as the potential molecular mechanism in esophageal squamous cell carcinoma (ESCC). MTT assay, clone formation experiment, wound healing assays, flow cytometry, Western blot and xenograft experiment were used to test the effects and molecular mechanism of SFN alone or combined with Akt/mTOR inhibitors on proliferation, migration, cell cycle phase, apoptosis of ESCC cells and tumor growth, respectively. The results showed that SFN significantly inhibited the viability and induced apoptosis of ECa109 and EC9706 cells in a dose-dependent manner by increasing the expression of the apoptotic proteins Cleaved-caspase 9. SFN combined with PP242, but not MK2206 and RAD001, had synergetic inhibition effects on the proliferation of ESCC cells. Moreover, the combination of SFN and PP242 had better inhibiting efficiency on clone formation, migratory, cell cycle phase and the growth of xenografts of ESCC cells, as well as the more powerful apoptosis-inducing effects on ESCC cells. Results of protein expression showed that PP242 abrogated the promotion effects of SFN on p-p70S6K (Thr389) and p-Akt (Ser473). These foundings demonstrated PP242 enhances the anti-tumor activity of SFN by blocking the activation of Akt/mTOR pathway by SFN in ESCC.

Introduction

Esophageal cancer (EC), a common gastrointestinal tumor, includes two main subtypes, esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC) [1]. The distribution of ESCC has obvious geographical characteristics with high incidence in East Asia, East and South Africa [2]. Surgical resection is currently the main treatment measure for early-stage ESCC, while patients with advanced ESCC have to suffer conservative treatment, such as radiotherapy and chemotherapy [3]. However, conventional chemotherapy drugs such as cisplatin exhibited highly toxicity and widespreadly adverse effects on patients [4]. Although targeted drugs promote the therapeutic efficiency of ESCC, tumor resistance and poor prognosis of patients are still not neglectful [5]. Thus, developing new drugs and exploring combined strategies of drugs might be desired for ESCC therapy.

Due to the low toxicity and side effects of plant-based drugs, In recent years, researchers have been keen to develop natural small molecules with antitumor effects from plants, such as curcumin, a polyphenolic compound isolated from spice turmeric [6] and dihydroartemisinin, a new anti-malarial drug isolated from Artemisia annua [7]. Recent studies have shown that cruciferous vegetables are rich in many phytochemicals with anticancer properties, such as Indole-3-carbinol (I3C) and SFN [8]. SFN has been demonstrated to have good efficacy against cardiovascular diseases [9], diabetes [10] as well as cancers such as breast cancer, prostate cancer and lung cancer, etc [11–13]. There are extensive molecular mechanisms about the anti-tumor effects of SFN [14]. For instance, SFN could inhibit the STAT3 pathway by inducing ROS production [15], activating Keap1/Nrf2 [14] and ERK1/2 [16] pathway and so on. In our previous study, we have demonstrated SFN induces cell autophagy and inhibiting autophagy enhances sulforaphane-induced apoptosis via targeting Nrf2 in ESCC [17]. Because Akt/mTOR pathway has the

critical regulating function to cell autophagy, in this study, we further explored effects and the potential mechanism of SFN alone or combined with Akt/mTOR pathway inhibitors on ESCC.

Materials And Methods

Reagent & antibodies

Sulforaphane, PP242, MK2206, RAD001 were purchased from Med Chem Express (Monmouth Junction, NJ, USA). Primary antibodies p-Rictor (Thr1135), Rictor, p-Akt (Ser473), Akt (pan), PRAS40, p-PRAS40 (Thr246), p-p70S6K (Thr389), p70S6K, Bax, Bcl-2, were purchased from Cell Signaling Technology (Danvers, MA, USA), and Cleaved-caspase 9 were acquired from Abcam (Cambridge, UK). The second antibody was obtained from Zhongshan Golden Bridge Biotechnology (Beijing, China).

Cell lines and animals

Human ESCC cell lines ECa109 and EC9706 purchased from Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) were cultured as described before [18]. 4–6 weeks athymic BALB/c male nude mice were purchased from Silikejingda Experimental Animal Ltd (Changsha, China) and fed in an individual ventilated caging (IVC) system with alternating 12h dark and 12h light. The operation of animals was agreed upon by the Animal Ethics Committee, Zhengzhou University and in accordance with the ARRIVE guidelines.

MTT assay

ECa109 and EC9706 cells (5×10^3 cells/well) seeded triply in 96-well plates were treated with different measures, and cell proliferation was detected by MTT assay as before [19]. When ECa109 and EC9706 cells were treated with SFN combined with MK2206, PP242 and RAD001, respectively, the combined Index (CI) was calculated according to the proliferation inhibition rate of cells by CompuSyn software. CI < 1 indicates drugs have synergistic effects, and the reverse indicates antagonism [20].

Colony formation assay

ECa109 or EC9706 cells seeded in 6-well plates at a density of 1×10^3 cells/well were treated with drugs and cultured for 10 days, cell colonies were fixed with 4% paraformaldehyde for 30 min, followed by being rinsed with PBS for 2–3 times and staining with 1% crystal violet (Beyotime, China), the number of colonies was subsequently counted by ImageJ software (NIH, Bethesda, MA, USA).

Wound healing assay

After ECa109 and EC9706 cells (3×10^5 cells/well) seeded in a 6-well plate were scratched a line with a sterile tip, cells were treated with 20 μM of SFN and 4 μM of PP242 alone or combined for 48 h, the migration distance was measured by NIS-Elements BR software under the microscope. The relative mobility ratio (%) = migration distance of cells in experiment group/migration distance of cells in control group × 100%.

Cell cycle assay

After ECa109 and EC9706 cells were treated with 20 μM of SFN and 4 μM of PP242 alone or combined for 48 h, the proportion of cells in different cell cycle phases were investigated by flow cytometry as before [18].

Cell apoptosis assay

After ECa109 and EC9706 cells (3×10^5 cells/well) were treated with different measures, cells were collected, washed, and resuspended in the binding buffer and stained with Annex-V and PI, cell apoptosis was investigated by a flow cytometer as our previous study [21].

Mitochondrial membrane potential (ΔΨm) assay

After ECa109 and EC9706 cells (3×10^5 cells/well) seeded in 6-well plates were treated with 20 μM of SFN and 4 μM of PP242 alone or combined for 48 h, cells were collected and incubated with JC-1 working solution (Beyotime Biotech, Shanghai, China) at 37°C for 20 min in the dark. Cells were washed with PBS and re-suspended with JC-1 staining buffer, and ΔΨm of cells was investigated by a Flow cytometer.

Western blot

30 μg of total protein abstracted from cells or xenograft tissues was separated using SDS-PAGE and transferred onto PVDF membrane, expression of proteins was detected by Western blot as before [18]. GAPDH was used as an internal control, the density of bands was analyzed by ImageJ software.

Animal experiment

After 20 nude mice were adaptively fed for one week, ECa109 cells (1×10^7 cells in 0.2 mL PBS) were inoculated hypodermically into the right forelimbs of the mice. When the average tumor volume reached 100–120 mm^3, the mice were divided randomly into four groups (5 mice/group), and the experimental group received an intraperitoneal injection of SFN (5 mg/kg) and PP242 (4 mg/kg) alone or combined every other day for 14 days. At the end of treatment, the mice were sacrificed and the tumor tissue was separated and weighed. A measure of tumor size and evaluation of the efficacy of the drugs as described previously [19].

H&E staining and in situ TUNEL assay

Tissues from mice fixed in a 4% paraformaldehyde solution and embedded in paraffin were sliced into 4–6 μm slides and subjected to hematoxylin and eosin (H&E) staining. Cell apoptosis in tumor tissues was detected by Transferase-mediated dUTP nick end labeling (TUNEL) kit as described before [22].

Statistical analysis

All experiments were repeated independently at least three times. The results were analyzed statistically by one-way analysis of variance (ANOVA) using GraphPad Prism 5 software. The data were expressed as mean ± standard deviation and $P < 0.05$ was considered as statistically significant.

Results

SFN inhibited ESCC but activated Akt/mTOR pathway

ECa109 and EC9706 cells were treated with 0, 1, 10, 20, 50 and 100 μM of SFN, the results of cell viability measured by MTT were shown in Fig. 1a, SFN inhibited the proliferation of ECa109 and EC9706 cells in a dose- and time-dependent manner, with IC_{50} values of 31.58 ± 1.50 and 14.95 ± 1.72 μM in ECa109 cells and 38.78 ± 1.59, 22.79 ± 1.36 μM in EC9706 cells at 48 and 72 h, respectively. The anti-proliferative effect of SFN was further demonstrated by the clone formation assay. As shown in Fig. 1b, SFN inhibited cell colony formation, with lower cell colony numbers (89 ± 26 and 67 ± 17 for ECa109 and 101 ± 21 and 87 ± 19 for EC9706 at 5 and 10 μM, respectively) than that in the control group (176 ± 32 for ECa109 and 202 ± 23 for EC9706) ($P < 0.05$). Apoptosis of ECa109 and EC9706 cells assayed by a flow cytometer was shown in Fig. 1c, the cell apoptosis rates were 6.93 ± 0.37%, 26.60 ± 0.73% and 36.63 ± 1.02% in ECa109 cells, and 5.00 ± 0.73%, 19.20 ± 0.57% and 29.37 ± 1.23% in EC9706 cells treated with 0, 10, and 20 μM of SFN, respectively, for 48 h, which had a statistical difference between the control group and the experimental group ($P < 0.01$), indicating that SFN induced apoptosis of ESCC cells. In addition, expression of proteins in apoptotic and Akt/mTOR pathway was detected by Western blot after ECa109 and EC9706 cells were treated with 20 μM of SFN for 0, 3, 6, 12 and 24 h, respectively, and the results showed that SFN promoted the expression of Cleaved-caspase 9, but not Bcl-2 and Bax (Fig. 2a). However, we found SFN activated Akt/mTOR pathway by promoting the phosphorylation of Akt (Ser473) and p70S6K (Thr389) (Fig. 2b). The results suggested that SFN inhibited ESCC through activating apoptotic pathways, while the activating effects of SFN on Akt/mTOR pathway might impair the anti-tumor efficiency of SFN.

Pp242 enhanced inhibitory effects of SFN on ESCC by inhibiting activation of SFN on Akt/mTOR pathway

Considering the activation of SFN on Akt/mTOR pathway, we speculated the combination of Akt/mTOR pathway inhibitors and SFN might have better anti-tumor effects. Therefore, cell proliferation was investigated after ESCC cells were treated with SFN (0, 10, 20 μM) combined with Akt/mTOR inhibitors MK2206 (0, 5, 10, 15 and 20 μM), PP242 (0, 0.5, 1, 5 and 10 μM) and RAD001(0, 5, 10, 20 and 30 μM) for 48 h, respectively, and the combination efficiency was judged according to the CI value. As shown in Fig. 3 and Table 1, CI > 1 in both ECa109 and EC9706 cells treated with SFN combined with RAD001; when cells were treated with SFN combined with MK2206, CI > 1 in ECa109 cells and CI < 1 in EC9706 cells; while CI < 1 in the two cell line cells treated with SFN combined with PP242, indicating that SFN combined with PP242 had better inhibiting efficiency on ESCC than combined with MK2206 or RAD001 ($P < 0.001$ or $P < 0.01$).

Table 1 CI of SFN combined with AKT/mTOR inhibitor on ESCC cells

	CI_{ECa109}		CI_{EC9706}	
	SFN(μmol/L)		SFN (μmol/L)	
	10	20	10	20
PP242 (μmol/L)				
0.5	0.83 ± 0.09	0.69 ± 0.26	0.14 ± 0.13	0.17 ± 0.09
1	0.72 ± 0.53	0.63 ± 0.25	0.17 ± 0.01	0.19 ± 0.02
5	0.56± 0.14	0.40 ± 0.02	0.24 ± 0.07	0.32 ± 0.11
10	0.45 ± 0.33	0.31 ± 0.09	0.43 ± 0.41	0.45 ± 0.11
MK2206 (μmol/L)				
5	1.39 ± 0.21	1.92 ± 0.23	0.42 ± 0.33	0.43 ± 0.11
10	1.06 ± 0.48	0.93 ± 0.47	0.66 ± 0.01	0.54 ± 0.24
15	1.03 ± 0.38	0.89 ± 0.27	0.77 ± 0.23	0.74 ± 0.20
20	0.83 ± 0.36	0.72 ± 0.30	0.90 ± 0.40	0.82 ± 0.50
RAD001 (μmol/L)				
5	1.80 ± 0.95	0.96 ± 0.29	1.25 ± 0.90	1.78 ± 0.35
10	1.27 ± 0.24	0.69 ± 0.30	1.16 ± 0.79	1.67 ± 0.10
20	1.12 ± 0.87	0.61 ± 0.46	1.17 ± 0.38	1.60 ± 0.10
30	0.58 ± 0.46	0.48 ± 0.09	0.94 ± 0.40	1.27 ± 0.11

To further demonstrate the combined effect of SFN and PP242, colony formation, migration, cell cycle and apoptosis of ECa109 and EC9706 cells treated with SFN and PP242 alone or combined were investigated. Compared to the control group, SFN and PP242 inhibited the formation of colonies ($P < 0.05$), while the combination of SFN and PP242 had stronger inhibition effects than that of the single-drug group (Fig. 4a, $P < 0.05$). Also, SFN and PP242 inhibited the migration of cells and retarded cells in the G_2 phase, especially when they were combined (Fig. 4b, c). PP242 had no obvious effects at the current concentration, while enhanced the apoptosis-inducing and JC-1 monomer-promoting effects of SFN on ESCC cells (Fig. 4d, e). The results above indicated that SFN combined with PP242 had better anti-tumor effects on ESCC.

To explore the molecular mechanism that PP242 combined with SFN inhibited ESCC, ECa109 and EC9706 cells were treated with 20 μM of SFN and 4 μM of PP242 alone or combined for 24 h, and expression of proteins in the Akt/mTOR pathway was shown as Fig. 4f. Compared to the corresponding

control group, SFN promoted but PP242 inhibited the expression of p-p70S6K (Thr389), p-Akt (Ser473) and p-PRAS40, while their expression was still inhibited when SFN combined with PP242 in ECa109 and EC9706 cells, indicated that PP242 blocked the activation of SFN on proteins of Akt/mTOR pathway in ESCC ($P < 0.001$ or $P < 0.01$, $P < 0.05$).

SFN combined with PP242 had stronger inhibiting effects on xenografts of ESCC

As shown in Fig. 5a, b, as the treatment time prolonging, the growth of tumors treated with SFN or PP242 was slower and the tumor volume was smaller than that in the control group, especially the combined group. Compared to the control group, apoptosis-related features such as cell shrinkage and nuclear merging, as well as more brown positive cells were observed in experimental groups, especially in the SFN + PP242 group (Fig. 5c, $P < 0.001$ or $P < 0.01$). The tumor inhibition rate in SFN + PP242 group is 56.51%, which was obviously higher than SFN (20.55%) or PP242 (39.97%) group, and the relative tumor growth rate (T/C%) in SFN + PP242 group was < 40% (Table 2). These results above indicated SFN and PP242 alone could inhibit the growth of xenografts in nude mice, while the combination of them had more significant efficiency. Results of protein expression were consistent with that in vitro, SFN stimulated expression of p-Akt (Ser473), p-p70S6K (Thr389) and p-PRAS40 (Thr246), and inhibited expression of p-Rictor (Thr1135), while PP242 weakened the activation of SFN on them (Fig. 6d).

Table 2
Efficiency of drugs on nude mice

Group	RTV	T/C (%)	Tumor weight (g)	Tumor inhibitory rate (%)
Control	12.61	-	1.42 ± 0.13	-
SFN	7.84	62.19	1.13 ± 0.11	20.55
PP242	7.03	55.78	0.85 ± 0.05	39.97
S + P	4.10	32.55*	0.62 ± 0.04	56.51*

Note: Relative tumor volume (RTV) = V_t / V_0, in them V_0 was tumor volume at the beginning of treatment, and V_t was tumor volume at every treatment). T/C% = TRTV/CRTV×100%, in them TRTV was the mean of RTV in experiment group during the treatment, and CRTV was the mean of RTV in the control group during the treatment). T/C% ≤ 40% and $P < 0.05$ were considered as effective treatment, T/C % > 40% was considered as invalid treatment.

The toxicity of drugs was primarily evaluated according to the body weight, H&E staining of live and kidney, blood routine, the indicators of liver and kidney function, as well as the organ coefficients of nude mice, and the results showed the data above had no obvious difference in each group (Fig. 6e, f, Table. 3–5), indicating that SFN and PP242 had no obvious toxicity at the current dose.

Discussion

In this study, we demonstrated SFN could inhibit the proliferation and induce apoptosis of ESCC cells. In addition, we demonstrated that mTOR pathway inhibitor PP242 could enhance the anti-tumor efficiency of SFN on ESCC by blocking the activation of SFN on Akt/mTOR pathway in vitro and in vivo.

Akt/mTOR pathway, as a crucial pathway in tumorigenesis and development, participates in various biological processes such as cell migration, apoptosis, and autophagy [23]. mTOR is a core regulator in the pathway and is involved in two multisubunit complexes, mTORC1 and mTORC2. mTORC1 could be activated by growth factor, chemokines and nutrients (glucose, amino acids) through PI3K/Akt pathway, thus regulating protein synthesis and cell growth by phosphorylating p70S6K, while mTORC2 mainly regulated actin cytoskeleton network through phosphorylating Akt at Ser473 site mediated by Rictor [18, 24]. Many Akt/mTOR pathway inhibitors have been developed to treat cancers [25]. RAD001 is an inhibitor of mTORC1 and has been approved by the FDA for treating many advanced malignancies [26]. MK2206, an allosteric Akt inhibitor, has high anti-tumor efficiency and can improve the chemotherapy regimen's anti-tumor effect [27]. while PP242 is an ATP-competitive mTORC1 and mTORC2 inhibitor, which has more powerful anti-proliferative and pro-apoptotic effects aspects than mTORC1 inhibitor [28].

Many studies have demonstrated the regulation effects of SFN on the PI3K/Akt/mTOR pathway, but the results are not consensus and even opposite. Some researchers have shown that SFN inhibits tumorigenesis by suppressing the PI3K/Akt/mTOR pathway [16, 29], while other studies demonstrate the activation of SFN on Akt/mTOR pathway [30, 31]. Our results in this study demonstrated that SFN activated Akt/mTOR pathway by promoting phosphorylation of Akt (Ser473) and p70S6K (Thr389) in ESCC (Fig. 2b), which may impair the anti-tumor effect of SFN. Thus, We explored firstly the efficiency of SFN combined with Akt/mTOR pathway inhibitor RAD001, MK2206 and PP242 on the proliferation of ESCC cells, respectively. Based on the CI value, we found that SFN combined with PP242 has better proliferation-inhibiting effects on ESCC cells than MK2206 and RAD001 (Fig. 3). We subsequently demonstrated the excellent combined efficiency of PP242 and SFN on migration, cell cycle phase and apoptosis of ESCC cells. The results of protein expression revealed that PP242 counteracted the activation of SFN to p-Akt (Ser473) and p-p70S6K (Thr389) and the inhibition to Rictor (Thr1135) (Fig. 4), which might be the molecular mechanisms that the combination of SFN and PP242 was more effective for treating ESCC.

Because the bioavailability and metabolism of SFN in vivo might impact the efficiency in fact, the effects in vitro might not represent completely the real efficiency of the drug, which must need further verification in vivo [32]. We, therefore, established xenografts models of ESCC cells using nude mice and investigated the inhibitory effect of SFN combined with PP242 on ESCC in vivo. The data showed that the small dose of SFN (5 mg/kg) and PP242 (4 mg/kg) alone had slight adverse effects on tumor growth, while SFN combined with PP242 could significantly suppress the growth of xenografts and induced cell apoptosis (Fig. 5a-c), and the molecular mechanism was consistent with that in vitro (Fig. 5d). Taken together, PP242 significantly increased the sensitivity of ESCC cells to SFN in vivo and in vitro.

Conclusion

In our current study demonstrated that SFN could inhibit ESCC development and had better anti-tumor effects on ESCC when combined with PP242, which might be related that PP242 retarded the activation of SFN on the Akt/mTOR pathway (Fig. 6).

Declarations

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Author contributions: Guiqin Hou and Zhaoming Lu conceived the study. Zhaoming Lu and Yalin Zhang wrote the manuscript. Yalin Zhang, Yujia Xu, Huiyun Wei, Yan Li conducted the experiments and analyzed the data. Guiqin Hou, Wen Zhao, and Pengju Wang secured the fundings. All authors agree to be accountable for all aspects of work ensuring integrity and accuracy.

Ethical approval: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Conflict of Interest The authors declare there is no conflict of interest in this study.

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Figures

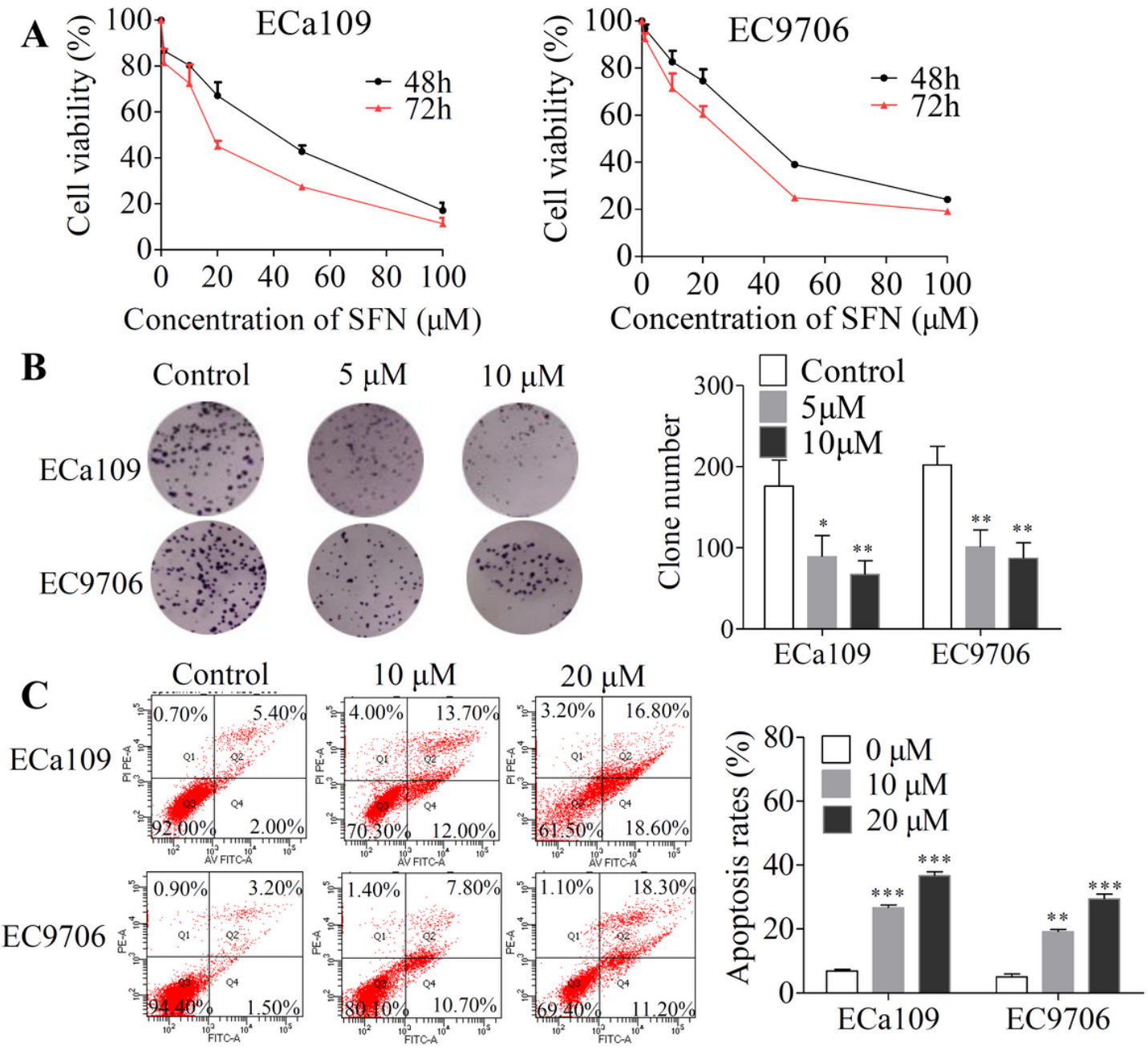

Figure 1

SFN inhibited proliferation and induced apoptosis of ESCC cells. a ECa109 and EC9706 cells were treated with SFN, and cell viability was detected by MTT assay. b Effect of SFN on colony formation of ECa109 and EC9706 cells. c ECa109 and EC9706 cells were treated with SFN for 72 h, cell apoptosis was measured by flow cytometry. *p < 0.05, **p < 0.01, ***p < 0.001, versus control group.

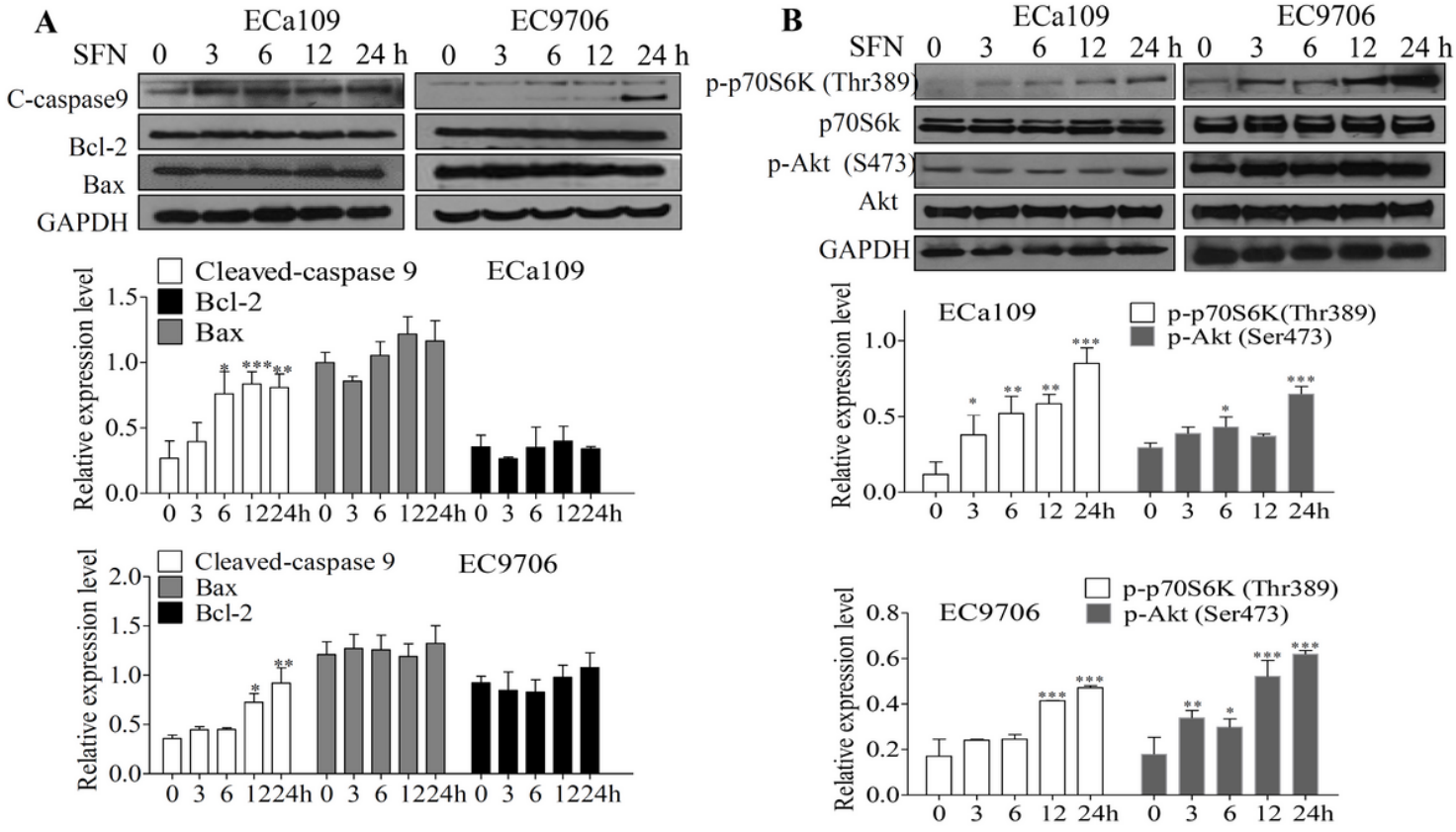

Figure 2

SFN activated apoptotic pathway and Akt/mTOR pathway. After ECa109 and EC9706 cells were treated with 20 μM SFN for 0, 3, 6, 12 and 24 h, expression of proteins in apoptosis pathway (A) and Akt/mTOR pathway (B) was investigated by Western blot. *p < 0.05, **p < 0.01, ***p < 0.001, versus control group.

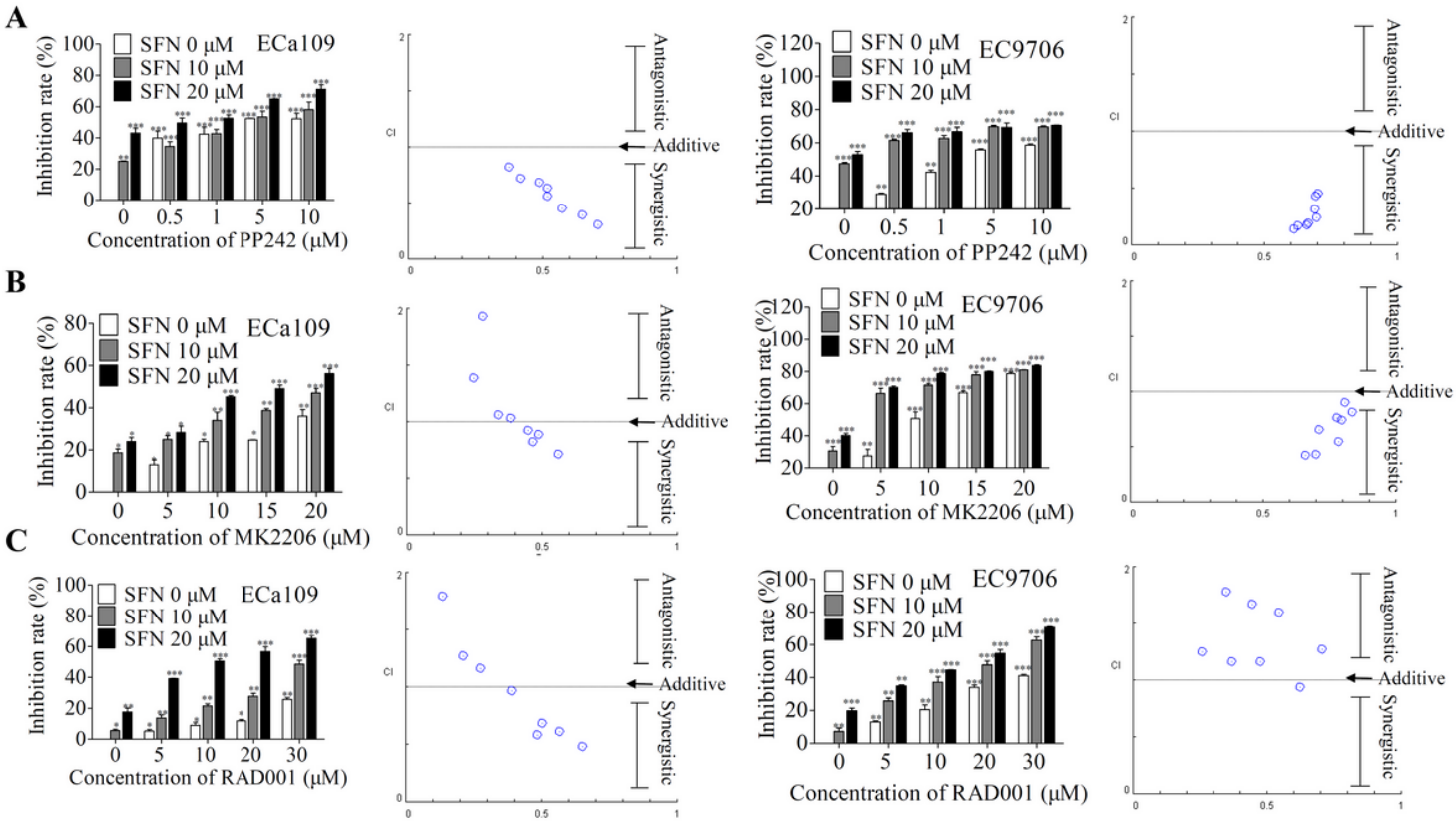

Figure 3

Effects of SFN combined with Akt/mTOR pathway inhibitors on proliferation of ESCC cells. After ECa109 and EC9706 cells were treated with SFN combined with PP242 (a), MK2206 (b) and RAD001 (c) for 48 h, cell proliferation was detected by MTT assay. CI was calculated by CompuSyn software and CI < 1 indicates the combined drugs have synergistic effects.

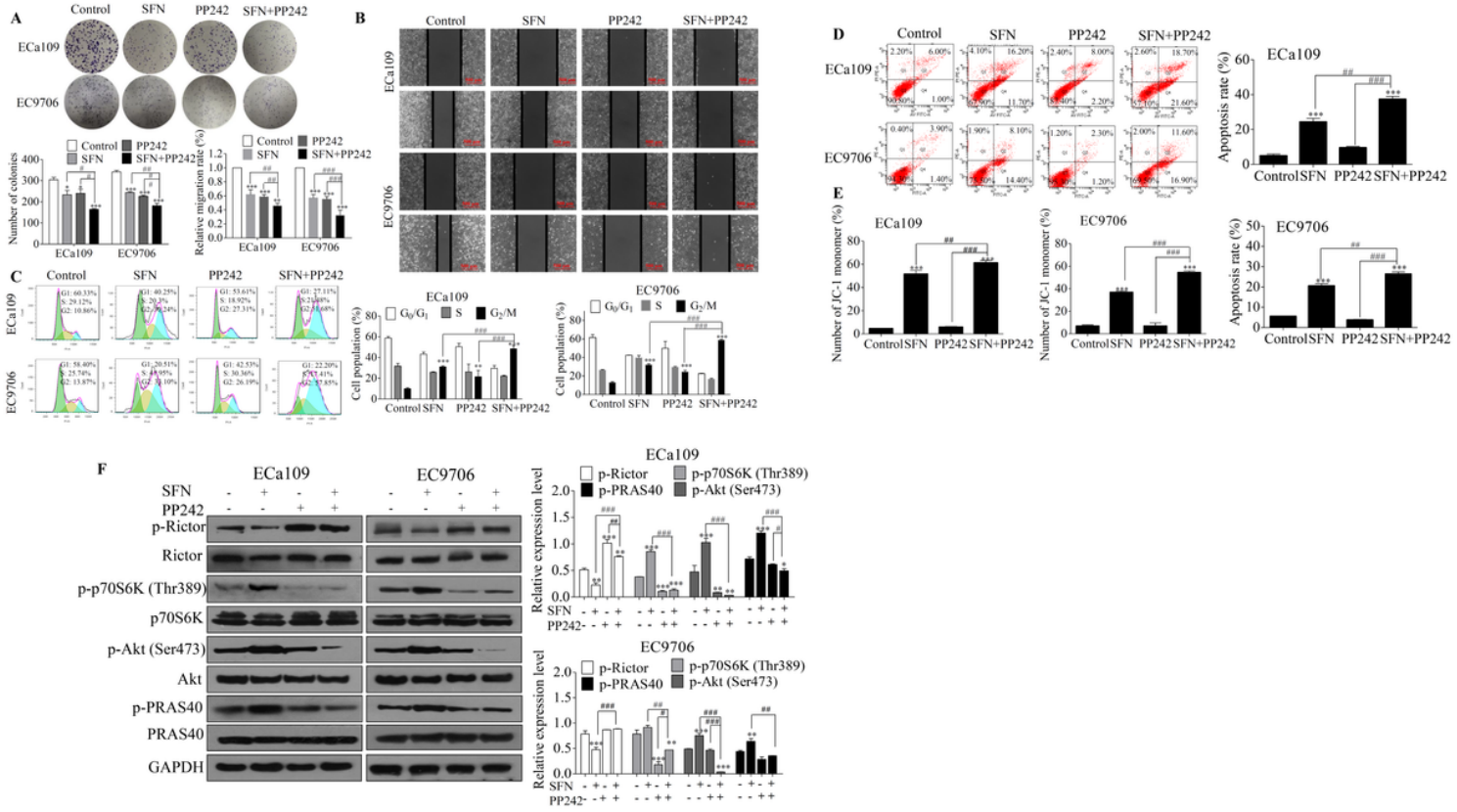

Figure 4

Inhibition effects and mechanism of SFN combined with PP242 on ESCC. a Colony information ability of ECa109 and EC9706 cells treated with 5 μM of SFN and 0.5 μM of PP242 alone or combined. Migration ability (b), cell cycle (c), and apoptosis (d) and JC-1 monomers (e) of ECa109 and EC9706 cells treated with 20 μM of SFN and 4 μM of PP242 alone or in combination for 48 h. f The expression of proteins in Akt/mTOR pathway of ECa109 and EC9706 cells treated with 20 μM of SFN alone or combined with 4 μM of PP242 for 24 h. *p < 0.05, **p < 0.01, ***p < 0.001, versus control group; #p < 0.05, ##p < 0.01, ###p < 0.001, versus single treatment drug.

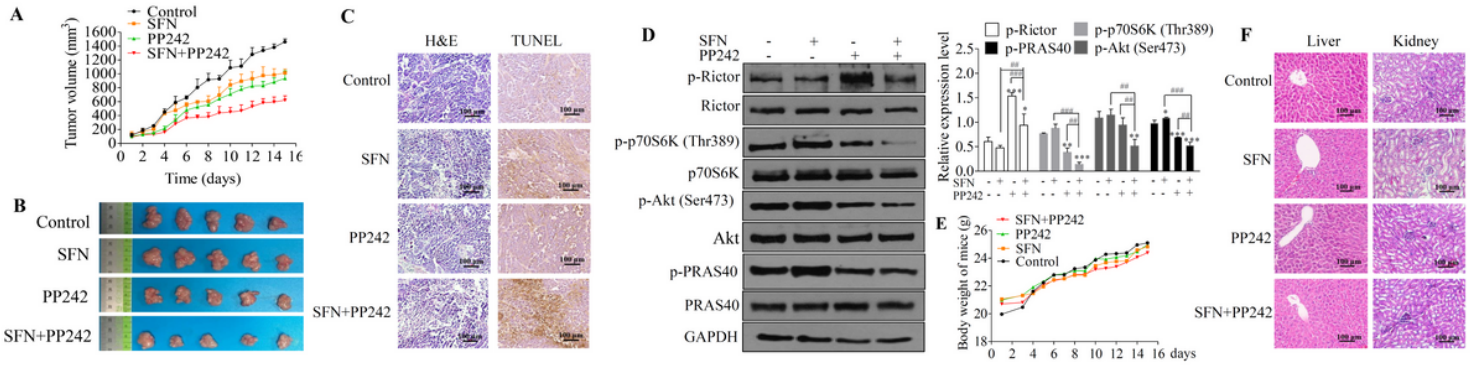

Figure 5

Effects of SFN and PP242 alone or combined on xenografts of ESCC cells (n=5). a Tumor growth curve. b Xenograft from in nude mice at the end of treatment. c H&E staining and TUNEL assay of tumor tissue. d

Expression of proteins in Akt/mTOR pathway in tumor tissues from mice. e The body weight curve of mice. f H&E staining of the liver and kidney tissues of mice. *p < 0.05, **p < 0.01, ***p < 0.001, versus control group; #p < 0.05, ##p < 0.01, ###p < 0.001, versus single treatment drug.

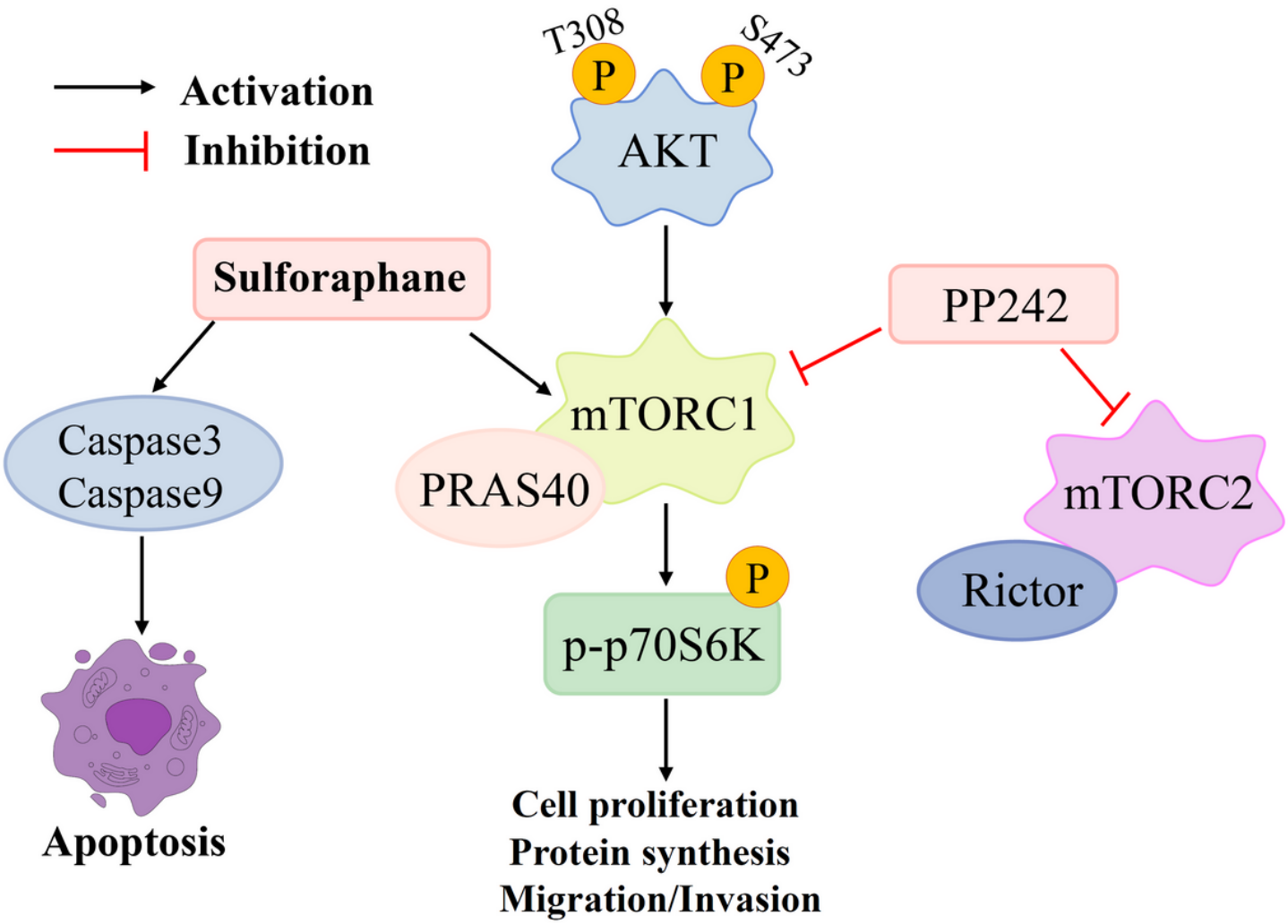

Figure 6

PP242 enhances the anti-ESCC effects of SFN by blocking the activation of SFN on the Akt/mTOR pathway.

Supplementary Files

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