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EGCG sensitizes chemotherapeutic-induced cytotoxicity by targeting the ERK pathway in multiple cancer cell lines

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Abstract

Epigallocatechin-3-gallate (EGCG), a major polyphenol component of green tea, presents anticancer efficacy. However, its exact mechanism of action is not known. In this study, we evaluated the effect of EGCG alone or in combination with current chemotherapeutics [gemcitabine, 5-flourouracil (5-FU), and doxorubicin] on pancreatic, colon, and lung cancer cell growth, as well as the mechanisms involved in the combined action. EGCG reduced pancreatic, colon, and lung cancer cell growth in a concentration and time-dependent manner. EGCG strongly induced apoptosis and blocked cell cycle progression. Moreover, EGCG enhanced the growth inhibitory effect of 5-FU and doxorubicin. Of note, EGCG enhanced 5-FU's and doxorubicin's effect on apoptosis, but not on cell cycle. Mechanistically, EGCG reduced ERK phosphorylation concentration-dependently, and sensitized gemcitabine, 5-FU, and doxorubicin to further suppress ERK phosphorylation in multiple cancer cell lines. In conclusion, EGCG presents a strong anticancer effect in pancreatic, colon, and lung cancer cells and is a robust combination partner for multiple chemotherapeutics as evidenced by reducing cancer cell growth, in part, by inhibiting the ERK pathway.

Keywords

pancreatic cancer; colon cancer; lung cancer; epigallocatechin-3-gallate; gemcitabine; 5-FU; doxorubicin; ERK

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Introduction

Pancreatic, colon, and lung malignancies have the highest cancer morbidity and mortality for both genders, in the United States [1]. Besides surgery and radiation, the use of chemotherapy, either alone or in combination, is one of the most common ways to treat cancer. Unfortunately, conventional drug therapies have obvious limitations due to chemoresistance, as well as undesirable systemic side effects, which are often severe. For example, gastrointestinal tumors treated with the chemotherapy 5-fluorouracil (5-FU), can easily acquire resistance. Furthermore, 5-FU is associated with health risks, ranging from nausea and diarrhea to neurological disorders and myelosuppression [2, 3]. For these reasons, it is imperative to search for safer treatment strategies.

Over the last two decades, there has been a growing interest in identifying bioactives with anticancer effects. Due to chemotherapy's significant side effects, combining chemotherapeutic drugs with other agents, such as bioactives, is a promising approach to reduce toxicity while maintaining (or enhancing) the desired efficacy. Among several bioactives under investigation, many phytochemicals have been shown to possess anticancer effects, suppressing cancer growth at various steps. Epigallocatechin-3-gallate (EGCG), a major bioactive component in green tea, is one of these phytochemicals with anticancer activity [4]. Indeed, we have recently shown that EGCG synergized with gemcitabine to suppress pancreatic cancer cell growth [5, 6]. However, the ability of EGCG to enhance the effect of chemotherapeutic drugs in other cancer types is not completely understood.

Raf/MEK/ERK pathway is frequently activated in various malignancies, correlating to cell growth, cell cycle, and even apoptosis prevention [7]. Notably, activation of Raf/MEK/ERK pathway is also correlated to drug resistance [8]. Thus, inhibitors of Raf, MEK, ERK or some downstream effectors could be the target for therapeutic intervention. However, though the Raf/MEK/ERK pathway plays a vital role in controlling tumor growth and drug resistance, the regulation effect of EGCG remains unclear.

In this study, we evaluated the efficacy and mechanisms of EGCG in combination with chemotherapeutics (gemcitabine, 5-FU, and doxorubicin) active against pancreatic, colon, and lung cancers to elucidate whether EGCG is a potential adjuvant agent for cancer treatment. We observed that EGCG enhanced gemcitabine, 5-FU, and doxorubicin cell growth inhibition and induced apoptotic cell death in pancreatic, colon, and lung cancer cells, and this effect was associated, in part, with the suppression of the Raf/MEK/ERK pathway.

Materials and Methods

Chemicals and Reagents

EGCG (≥98%) was purchased from Tocris (Minneapolis, MN) and a stock solution (100 mM) was prepared in sterile DMSO. Doxorubicin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (≥97.5%), RIPA lysis buffer, Halt Protease Inhibitor Cocktail, and Phosphatase Inhibitor Cocktail were purchased from MilliporeSigma (St. Louis, MO). SuperSignal™ West Dura Extended Duration Substrate were purchased from

ThermoFisher Scientific (Waltham, MA). Gemcitabine was purchased from BIOTANG (Waltham, MA, USA). 5-FU (≥99%) was purchased from Alfa Aesar (Haverhill, MA, USA). Bradford protein assay reagent, 30% (w/v) Acrylamide/Bis Solution, 4×Laemmli sample buffer, Immun-Blot Polyvinylidene difluoride (PVDF) Membranes and were purchased from Bio-Rad (Hercules, CA).

Cell Culture

Human pancreatic cancer cell lines (Panc-1, MIA PaCa-2, and BxPC-3), human colon cancer cell lines (SW480, HCT15, and HT29), and human lung cancer cell lines (HT1975, H358, and A549) were purchased from the American Type Culture Collection (Manassas, VA). All cell lines were grown as monolayers in the specific medium suggested by the vendor. Although these cells lines were not authenticated in our lab, they were characterized by cell morphology and growth rate, and cultured in our laboratory less than six months after being received.

Cell Viability

After treating cells with EGCG alone or together with specific chemotherapeutic drugs for 24, 48 and 72 h, the reduction of MTT dye was determined according to the manufacture's protocol (MilliporeSigma, St. Louis, MO).

Clonogenic Assay

This was performed as previously described [9]. Briefly, HCT15 colon cancer cells were plated in 6-well plates (1,000 cells per well), and treated with 5-FU alone or in combination with EGCG for 24 h. Following treatment, cells were then incubated with fresh media for 20 days. Media was replaced once weekly during the incubation. On the last day, colonies were fixed with methanol and stained with 0.1% (w/v) crystal violet in phosphate buffered saline (PBS) (pH 7.4). Cells were then rinsed with distilled water, air-dried, and colonies were counted and analyzed using ImageJ software (V1.46, NIH, Bethesda, MD, USA).

Cell Apoptosis

Cells were seeded in 100 mm plates at a density of 1.5 million cells per plate. The following day, cells were treated with EGCG, chemotherapy drugs, or a combination. After 48 h treatment, cells were trypsinized and stained with Annexin V-fluorescein isothiocyanate (FITC) (100× dilution) and propidium iodide (PI) (0.5 μg/mL) for 15 min. Annexin V-FITC and PI fluorescence intensities were analyzed by FACScan (Becton Dickinson, San Jose, CA, USA). Annexin V (+)/PI (−) cells are apoptotic cells, Annexin V (+)/ PI (+) cells have undergone secondary necrosis, and Annexin V (−)/ PI (+) cells are necrotic cells. Results were analyzed by using FlowJo software.

Cell Cycle Analysis

Cells were seeded in 6-well plates and treated the following day with EGCG, chemotherapy drugs, or a combination for 24 h. After each treatment, cells were trypsinized and fixed in 70% ethanol overnight at −20°C, stained with PI (50 μg/ml) and RNase A (10 mg/ml) for 15

min and subjected to flow cytometric analysis by FACScan (Becton Dickinson; San Jose, CA).

Western Blot

Following treatment with EGCG, chemotherapy drugs, or a combination, cells were lysed, and total cell fractions were obtained as previously described [10]. Aliquots of total fractions containing 10–30 μg protein were separated by using 10–12% (w/v) polyacrylamide gel electrophoresis and electroblotted to PVDF membranes. After blocking with 5% (w/v) non-fat milk for 1 h, membranes were probed overnight with the following primary antibodies (1:1000 dilution) from Cell Signaling Technology (Danvers, MA): Caspase-3 (Cat #14220), Caspase-7 (Cat #12827), Caspase-9 (Cat #9508), PARP (Cat #9542), phospho-Chk1 (Ser345) (Cat #2348), phospho-p53 (Ser15) (Cat #9286), p53 (Cat #2527), p21 Waf1/Cip1 (Cat #2947), cdc2 (Cat #28439), Cyclin B1 (Cat #12231), Bcl-xL (Cat #2802), Bad (Cat #9239), XIAP (Cat # 14334), survivin (Cat # 2808), p-ERK1/2 (Cat #4370), and ERK1/2 (Cat #9102). β-Actin (Cat #8457) was used at the same time as a loading control. After incubation for 60 min at room temperature in the presence of the secondary antibody (HRP-conjugated; 1:5,000 dilution), the conjugates were developed and visualized using a Molecular Imager FX™ System (BioRad; Hercules, CA) and analyzed using ImageJ software(V1.46, NIH, Bethesda, MD, USA).

Immunohistochemistry

Immunohistochemistry was performed using tumor samples from a previous efficacy study that evaluated the effect of EGCG and gemcitabine on murine pancreatic cancer xenografts [5]. Briefly, immunohistochemical staining for p-ERK1/2 (Cat #4370; Cell Signaling Technology, Danvers, MA, USA) was performed as previously described [33]. Briefly, paraffin-embedded sections (5 μm thick) were deparaffinized and rehydrated, followed by antigen retrieval performed by microwave-heating in 0.01 M citrate buffer (pH 6.0). H_2O_2 3% was used to block endogenous peroxidase activity for 10 min at room temperature. Slides were blocked for 60 min with serum, and incubated with primary antibody overnight at 4 °C. The following morning, slides were washed thrice with PBS, and then incubated with the biotinylated secondary antibody and the streptavidin-biotin complex (Invitrogen, Carlsbad, CA, USA) for 1 h each at room temperature. After washing with PBS three times, slides were stained with 3,3′-Diaminobenzidine tetrahydrochloride hydrate (DAB) solution, and then counterstained with hematoxylin. Images were captured at 100× magnification. At least five fields per sample were scored and analyzed using Image J software (V1.46, NIH, Bethesda, MD, USA).

Statistical Analysis

The data, obtained from at least three independent experiments, were expressed as mean ± standard deviation (SD). Statistical evaluation was performed using one-factor analysis of variance (ANOVA) followed by the Duncan test for multiple comparisons. T-tests were used to analyze the difference between two groups. A P value<0.05 was regarded as statistically significant.

Results

EGCG reduces cancer cell growth in multiple cancer cell lines

To test the anticancer effect of EGCG on cancer cell growth, we included nine human cancer cell lines from pancreatic (Panc-1, MIA PaCa-2, and BxPC-3), colon (HCT15, SW480, and HT29) and lung cancer (A549, H358, and HT1975) and treated them with increasing concentrations of EGCG (20–100 μM) for 24, 48, and 72 h. In all nine cell lines, EGCG reduced cancer cell growth in a time- and concentration-dependent manner. However, different cell lines displayed varying sensitivity to EGCG, as BxPC-3, HT1975, and HCT15 were relatively more sensitive to EGCG, while Panc-1, H358, and HT29 showed more resistance (Fig 1). The Inhibitory Concentration at 48 h (48 h-IC_{50}) for EGCG in each cell line is summarized in Figure 1D. Given the high prevalence of *Kras* mutations in pancreatic, colon, and lung cancer, we chose Panc-1, MIA PaCa-2, HCT15 and A549 cell lines, which are *Kras* mutant cell lines, for the subsequent studies.

EGCG reduces cancer cell growth through a strong cytokinetic effect

EGCG inhibited tumor growth through a potent cytokinetic effect. Treatment of Panc-1 and MIA PaCa-2 cells with EGCG for 48 h led to a concentration-dependent induction of apoptosis (Fig. 2A). EGCG at 1xIC_{50} for 48 h induced apoptosis by 3.5 and 2.1-fold over control in Panc-1 and MIA PaCa-2 cells, respectively ($p<0.01$). Notably, EGCG predominantly induced apoptotic cell death, with no significant induction of cell necrosis [Annexin V(−) but PI (+)].

These findings were validated by determining the activation and levels of apoptotic-related Caspases by microscopy and Western blot (Fig. 2B–C). In Panc-1 and MIA PaCa-2 cells, EGCG treatment induced the activation of Caspase 9, 7, and 3 in a concentration-dependent manner. For example, EGCG at 1xIC_{50} activated Caspase 3 levels by 2.9 and 3.0-fold in Panc-1 and MIA PaCa-2 cells, respectively, compared to the control group ($p<0.01$ for both). As a consequence of Caspase 3 activation, levels of cleaved poly(ADP-ribose) polymerase (PARP) increased in all EGCG treatments (Fig. 3C).

Next, we evaluated whether EGCG can also induce apoptosis in colon and lung cancer cells. For this purpose, we treated HCT15 colon cancer and A549 lung cancer cell lines with increasing concentration of EGCG for 48 h, and determined apoptotic-related caspases by Western blot. EGCG treatment induced the activation of Caspase 9, and PARP in a concentration-dependent manner in both HCT15 and A549 cells (Fig. 2D).

To explore the apoptosis mechanism induced by EGCG, we determined, in Panc-1 and MIA PaCa-2 cells, the expression levels of multiple proteins that regulate apoptosis, including proteins in the inhibitor of apoptosis protein (XIAP) and Bcl-2 family. As shown in Figure 3, while EGCG reduced Bcl-xl, XIAP, and survivin levels, it increased the levels of the proapoptotic protein Bad concentration-dependently.

Because ERK1/2 has been shown to modulate cell survival through the regulation of Bcl-2 protein family [11], we next evaluated the effect of EGCG on ERK phosphorylation. In

Panc-1 and MIA PaCa-2 cells, EGCG treatment for 24 h reduced ERK1/2 phosphorylation in a concentration-dependent manner (Fig. 3B).

Next, we evaluated whether EGCG can also modulate the ERK pathway in colon and lung cancer cells. For this purpose, we treated HCT15 colon cancer and A549 lung cancer cell lines with increasing concentration of EGCG for 24 h, and determined ERK phosphorylation by Western blot. Consistent with our findings in pancreatic cancer cells, EGCG treatment strongly reduced ERK phosphorylation in both HCT15 and A549 cells (Fig. 3C).

To examine whether EGCG can affect cell cycle progression, we performed flow cytometry to test cell cycle distribution and determined the levels of cell cycle regulators by Western blot. EGCG induced an S/G_2 arrest in Panc-1 cells. However, under the same experimental conditions, a lesser effect was observed in MIA PaCa-2 cells (Fig. 4A). Given the effect of EGCG on the S/G_2 transition, we examined the expression levels of G_2 phase checkpoint proteins. In Panc-1 and MIA PaCa-2 cells, EGCG treatment increased the expression of p-Chk1, p-p53, and p21 Waf1/Cip1, whereas it reduced the levels of cdc2 and Cyclin B1 (Fig. 4B).

EGCG enhances the cytotoxicity of chemotherapeutics in colon and lung cancer cells

The use of drugs in combination to treat cancer patients is a common practice. We have recently documented that EGCG enhances the chemotherapeutic efficacy of gemcitabine in pancreatic cancer cells and xenografts [5, 6]. Here, we evaluated whether EGCG can enhance the efficacy of chemotherapeutic drugs in colon and lung cancer cells. For this purpose, we treated cells with EGCG together with 5-FU or doxorubicin, two chemotherapeutics commonly used clinically and experimentally in colon and lung cancer. As shown in Figure 5A, EGCG increased the cytotoxicity of 5-FU in HCT15 cells. Compared with the control group, 20 μM 5-FU decreased cell growth to 62.7%, while the cell growth was further reduced to 29.4% after treatment together with EGCG at $1xIC_{50}$ ($p<0.01$). In A549, after co-treating cells with EGCG and doxorubicin, cell growth decreased to about 40%, lower than the doxorubicin alone treated groups, while kept a similar level as EGCG alone group (Fig. 5A).

In agreement with the growth inhibitory results, EGCG and 5-FU together also effectively inhibited the colony formation in HCT15 cells (Fig 5B). For example, 5-FU alone reduced the formation rate by 66.9%, and the inhibitory effect was enhanced to 90.1% when combined with EGCG ($p<0.01$).

EGCG and chemotherapy drugs together also induced more apoptosis compared with each treatment alone. In HCT15 and A549 cells, compared to control, treatment with 5-FU or doxorubicin for 48 h resulted in a 1.7 and 2.8 fold increase in apoptosis, respectively. The effect was further enhanced to 2.1 and 3.4 fold after treatment together with EGCG ($p<0.01$, Fig. 5C). In contrast, EGCG treatment did not enhance the cell cycle arrest inhibitory effect of 5-FU or doxorubicin on cell cycle progression (Fig. 5D).

EGCG enhances drug sensitivity by the inhibition of Raf/MEK/ERK pathway

We next investigated the potential mechanisms by which EGCG plus chemotherapeutics reduced cell growth and induced cell death by apoptosis. Because the ERK pathway plays a critical role in controlling tumor growth and drug resistance [12], we evaluated the effect of EGCG in combination with gemcitabine, 5-FU, and doxorubicin on the ERK pathway.

We first explored the effect of EGCG in combination with gemcitabine on ERK activation in pancreatic cancer cells. As shown in Figure 6A, EGCG 1xIC_{50} reduced the levels of ERK phosphorylation in Panc-1 and MIA PaCa-2 cells by 53% and 37.5% ($p<0.05$ for both), respectively, and this effect was enhanced in both cell lines, when combined with gemcitabine (70% and 49% in Panc-1 and MIA PaCa-2 cells, respectively). Of note, gemcitabine alone did not affect ERK phosphorylation in Panc-1 and MIA PaCa-2 cells (Fig. 6A). Consistent with the *in vitro* results, EGCG plus gemcitabine had an additive effect reducing the levels of p-ERK ($p < 0.05$) in pancreatic tumor xenografts [5] by 91%, compared to control, and the effect was stronger than either treatment alone (Fig. 6B).

Because ERK is known to phosphorylate STAT3 at the serine 727 residue [13], we also tested the effect of EGCG alone, gemcitabine alone, and EGCG and gemcitabine in combination on STAT3 phosphorylation. EGCG alone reduced STAT3 phosphorylation in Panc-1 and MIA PaCa-2 cell lines. While gemcitabine alone only reduced p-STAT3 levels in Panc-1 cells lines, the combination of EGCG plus gemcitabine had an additive effect and reduced STAT3 phosphorylation. In MIA PaCa-2 cells, p-STAT3 expression in the EGCG plus gemcitabine group was similar to that of EGCG alone (Fig. 6A).

We then evaluated whether EGCG enhanced the effect of 5-FU and doxorubicin on ERK phosphorylation in colon and lung cancer cells. In HCT15 cells, EGCG reduced ERK phosphorylation by 80% and no additional effect was observed when combined with 5-FU (Fig. 7A). In contrast, EGCG treatment reduced ERK phosphorylation in A549 by 27% and this was significantly enhanced when combined with doxorubicin (75% reduction vs. control; Fig. 7B).

Finally, in both HCT15 and A549 cells, the expression of Bcl-xl and XIAP decreased in the EGCG plus 5-FU as well as in the EGCG plus doxorubicin groups compared to 5-FU alone or doxorubicin alone (Figure 7A–B).

Discussion

Patients receiving chemotherapy usually experience side effects, many of which are often severe. Therefore, there is an active search for safer and more effective therapeutic approaches. In this study, we show that the polyphenol EGCG, is a successful combination partner of various chemotherapy drugs in pancreatic, colon, and lung cancer cells, and that its anticancer effect is due, in part, through the modulation of the ERK pathway.

EGCG is a major bioactive component in green tea, with strong anticancer activity in multiple types of cancers [6, 14–19]. Indeed, EGCG strongly reduced the growth of pancreatic, colon, and lung cancer cell in a time- and concentration-dependent manner. The

anticancer effect of EGCG results from its strong cytokinetic effect: inhibition of proliferation, induction of apoptosis, and block at the S/G_2 cell cycle transition [18]. The apoptotic effect of EGCG seems to be the dominant one. For example, EGCG at $1xIC_{50}$ induced apoptosis in pancreatic cancer cells by up to 3.5-fold compared to controls, with arrest of the cell cycle showing only a moderate effect. The apoptotic cascade of the pancreatic cancer cells was manifested by the activation of execution caspases [20], and the modulation of Bcl-2 and the inhibitor of apoptosis protein families by EGCG. The apoptotic effect was not restrained to only pancreatic cancer cells, since EGCG also strongly induced apoptosis in colon and lung cancer cells, showing that this effect is observed in multiple cancer types. Our findings are consistent with others, showing that EGCG strongly induces apoptosis in various types of tumors [21–24].

Conventional chemotherapy is commonly associated with both acute and chronic toxicity [25]. Based on the World Health Organization classification, signs of toxicity can be classified in grades 1–4 (ranging from mild (grade 1) to life-threatening (grade 4). Common side effects of gemcitabine, 5-FU, and doxorubicin include anorexia, nausea, vomiting, and fatigue. Other, less common but often severe, unwanted effects of these drugs include hair loss and low white blood cell count. For this reason, combining chemotherapy with safer agents, such as bioactives, is a viable option to potentially reduce side effects while maintaining or enhancing anticancer efficacy.

Over the past decades, there has been increasing interest in exploring the use of phytochemicals that be used as combination partners with chemotherapeutics [26]. For example, curcumin has been shown to enhance the efficacy of multiple chemotherapeutic drugs [27–29]. For example, it potentiates the effect of 5-FU in colon cancer cells by inhibition of NF-κB and Src protein kinase [29], and enhances the anticancer effect of gemcitabine in preclinical models of pancreatic cancer [28].

Besides curcumin, resveratrol, a polyphenol present in grapes and red wine, has strong antitumor effects [30–35]. Resveratrol has also been shown to be an effective combination partner with chemotherapy drugs [36]. For example, resveratrol has also been shown to sensitize pancreatic cancer and colon cancer to gemcitabine and 5-FU [37, 38]. Moreover, resveratrol has been shown to protect against the myotoxicity of doxorubicin in aged mice [39].

Specifically for EGCG, we have recently shown that EGCG is a strong combination partner with gemcitabine in pancreatic cancer cells and xenografts [5]. In addition, EGCG is a strong combination partner for 5-FU and doxorubicin in colon and lung cancer cells, respectively. Consistent with our findings, EGCG is also a great partner for many other drugs, including cisplatin [40], paclitaxel [41] and metformin [42], highlighting its translational potential. Of note, the differential sensitivity that the multiple cancer cell lines had to EGCG and the combination treatment could be due to different genetic mutations present in these cancer cell lines, making them, either, more resistant or more sensitive to treatment. Therefore, implementing high-throughput drug screening and single-cell profiling techniques [43, 44] that can rapidly find effective drug combination for cancers with specific

mutations, will likely be instrumental in improving cancer care and facilitating personalized treatment.

Mechanistically, ERK signaling pathway appears to an important pathway modulated by EGCG alone or in combination. The RAS-regulated RAF-MEK-ERK signaling pathway is frequently activated in various malignancies, correlating to cell growth, cell cycle, and even apoptosis prevention [7]. Moreover, activation of Raf/MEK/ERK pathway is also correlated to drug resistance [8]. The importance of this signaling pathway has driven the development of a variety of pharmaceutical agents to inhibit RAF/MEK/ERK axis in cancer and some RAF and MEK inhibitors are already approved and used in the clinic [45].

However, there is now much interest in targeting ERK directly for multiple reasons. A critical one is the development of acquired resistance to RAF or MEK inhibitors (i.e. KRAS or BRAF amplification, MEK mutation, etc.), which involves relief of negative feedback and pathway re-activation with all signaling going through ERK. This validates the search for ERK inhibitors with RAF or MEK inhibitors as an up-front combination. EGCG strongly reduces ERK phosphorylation and its downstream STAT3 activation. Furthermore, its effect on ERK phosphorylation is enhanced when combined with chemotherapy drugs, suggesting a key pathway is affected.

Although EGCG has shown promise in preclinical models of cancer, its use in the clinic has been limited, due, in part, to its poor bioavailability and stability. A few studies have shown a benefit of EGCG clinically in cancer therapy and prevention [46–48], as well as in ameliorating side effect from drugs and radiation [49, 50]. These above mentioned limitations have led to the exploration of multiple approaches, including the formulation of EGCG in nanoparticles, delivering it as a pro-drug, or using it in combination [44, 51–54]. All of these strategies are aimed at improving EGCG’s bioavailability and stability, with the ultimate goal of improving the clinical use of EGCG.

In summary, our study provides new insights into the cellular mechanisms responsible for the antitumor effect of EGCG when combined with chemotherapeutics in multiple cancer types. EGCG has a beneficial effect when combined with chemotherapeutics, in part, through the inhibition of the ERK pathway. Further studies are warranted to precisely assess the *in vivo* effects of EGCG in combination with chemotherapeutic drugs.

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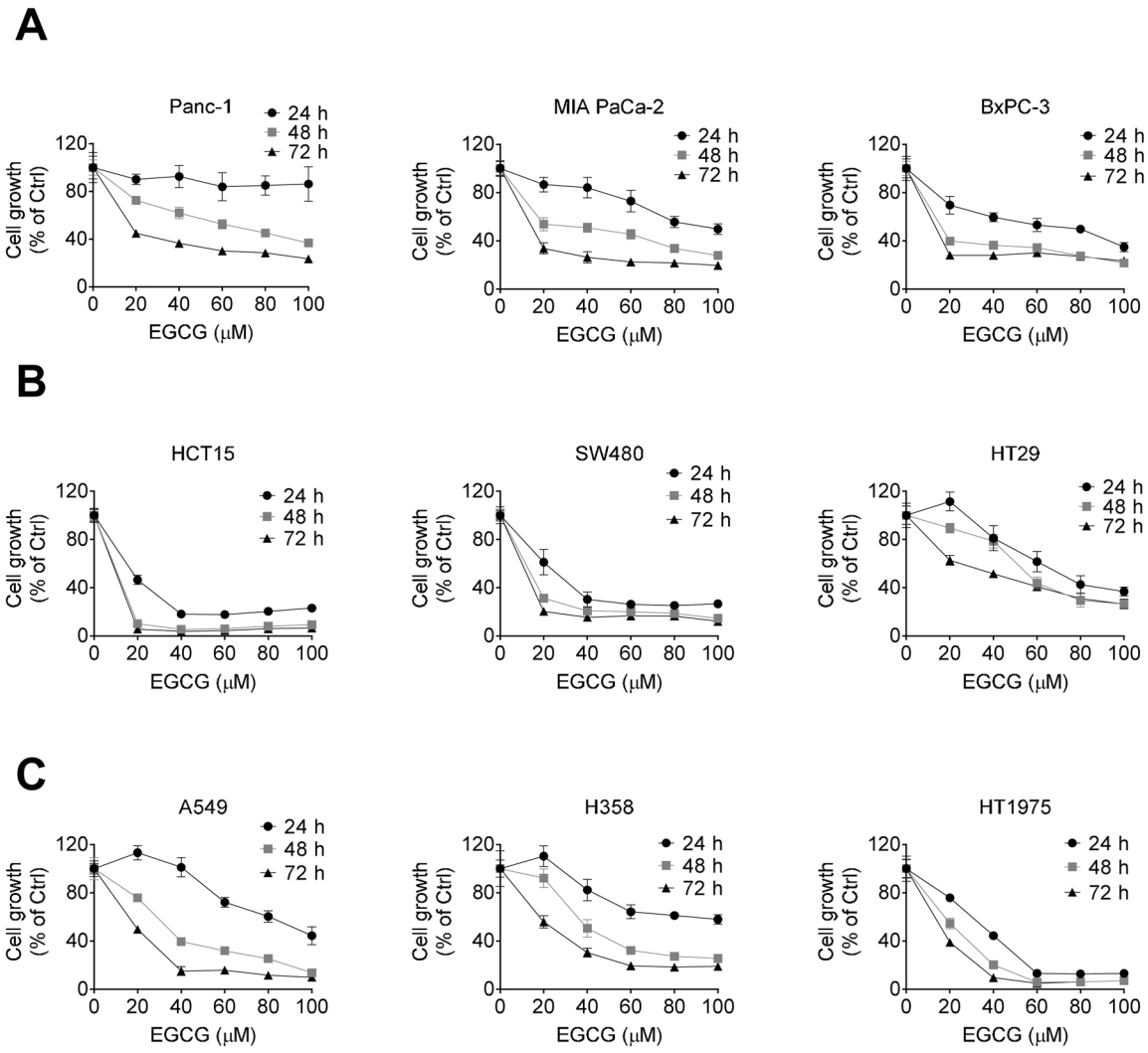
Highlights

- EGCG reduces cell growth and induces apoptosis in multiple cancer cell lines
- EGCG potentiates the growth inhibitory effect of chemotherapy drugs
- ERK mediates, in part, the anticancer effect of EGCG plus chemotherapy

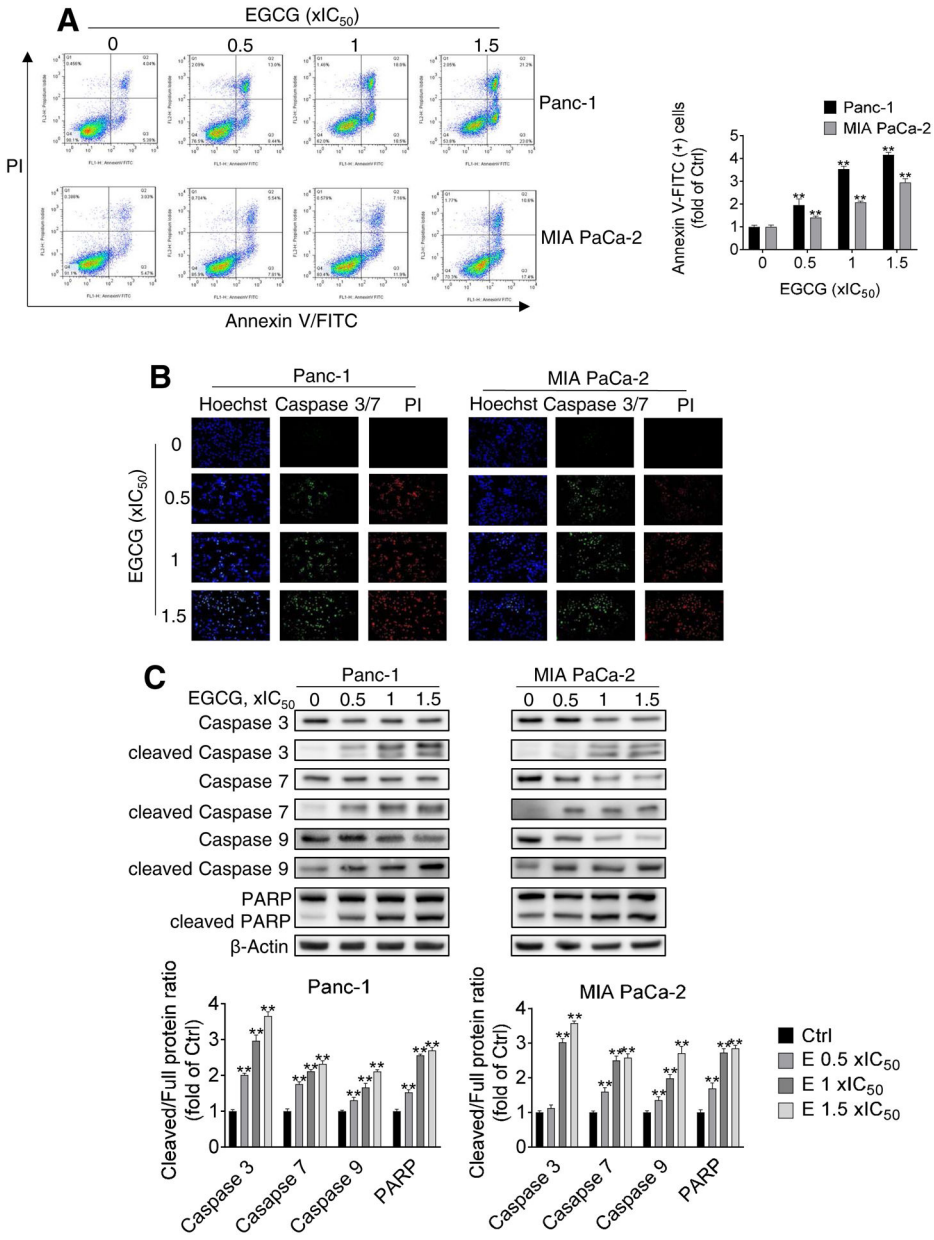


D

48 h-IC_{50}, μM								
Panc-1	**MIA PaCa-2**	**BxPC-3**	**HCT15**	**SW480**	**HT29**	**A549**	**H358**	**HT1975**
62.8±2.5	32.4±5.5	9.8±4.1	0.3±0.2	4.5±1.4	59.1±3.1	36.0±2.4	45.9±4.0	21.6±0.9

Figure 1: EGCG reduces pancreatic, colon, and lung cancer cell growth *in vitro*.

EGCG inhibits pancreatic, colon, and lung cancer cell growth in a concentration- and time-dependent manner. Cell growth was determined in: **A:** pancreatic Panc-1, MIA PaCa-2, BxPC-3, **B:** colon HCT15, SW480, HT-29, and **C:** lung A549, H358, and HT1975 cancer cells, after treatment with escalating concentrations of EGCG for 24, 48 or 72 h. Results (mean ± SD) are expressed as percentage of control. **D:** Inhibitory Concentration 50 (IC_{50}) values for pancreatic, colon, and lung cancer cells treated with EGCG for 48 h (mean ± SD).



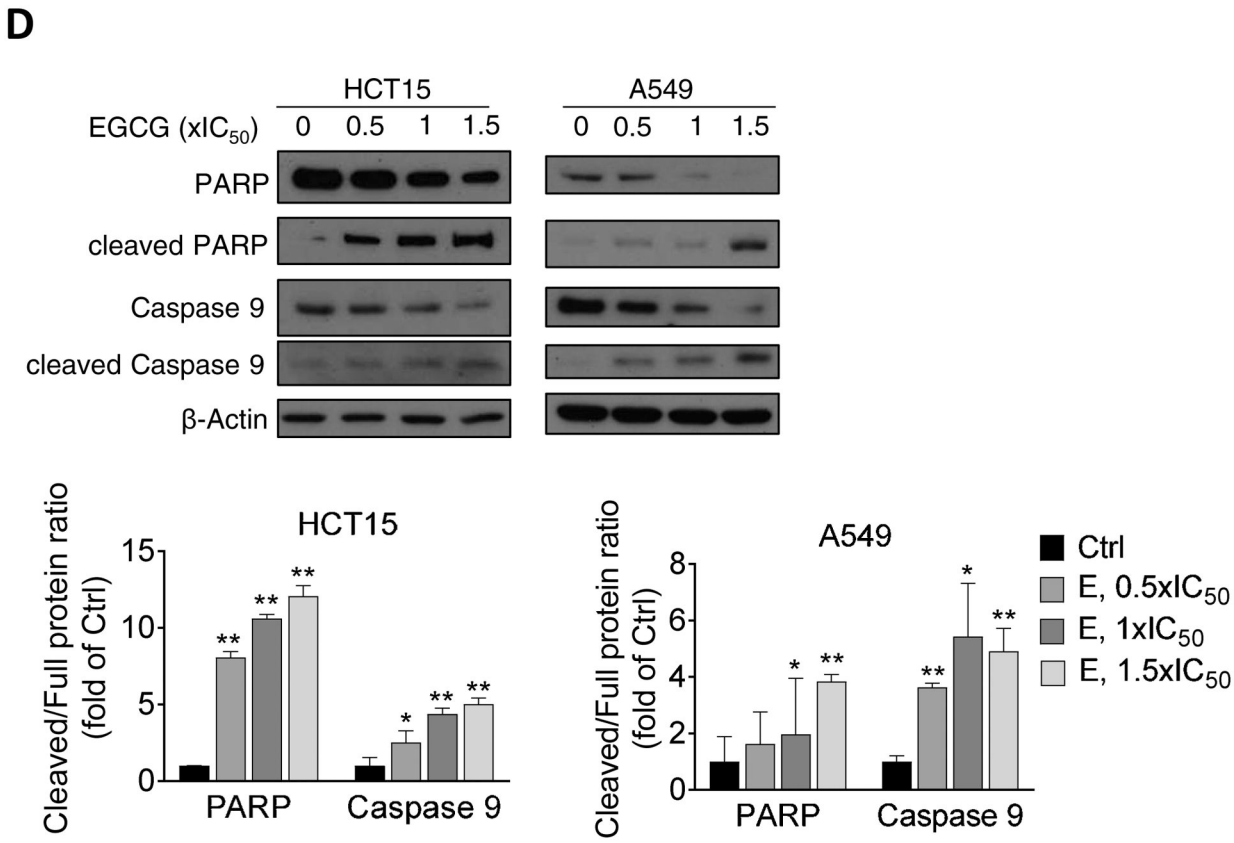


Figure 2: EGCG induces cell death by apoptosis in pancreatic, colon, and lung cancer cells.

A: Panc-1 and MIA PaCa-2 cells, treated with EGCG for 48 h, were stained with Annexin V/propidium iodide (PI), and the percentage of apoptotic cells was determined by flow cytometry. Results are expressed as fold change of control. *$p<0.05$, **$p<0.01$ *vs. control.* **B:** Caspase 3/7 activation was examined by immunofluorescence, by triple-staining control and EGCG-treated cells with Hoechst, PI and Caspase 3/7 green detection reagents. Representative images are shown (200x). **C:** Immunoblots for full length and cleaved caspases 3,7, and 9 as well as full length and cleaved PARP in total cell protein extracts from Panc-1 and MIA PaCa-2 cells treated with escalating concentrations of EGCG (0.5x, 1x and 1.5x IC_{50}) for 48 h. Loading control: β-Actin. Bands were quantified and results are shown as the ratio between the cleaved/full length protein; *$p<0.05$, **$p<0.01$ *vs. control.* **D:** Immunoblots for full length and cleaved caspase 9 as well as full length and cleaved PARP in total cell protein extracts from HCT15 colon cancer and A549 lung cancer cells treated with escalating concentrations of EGCG (0.5x, 1x and 1.5x IC_{50}) for 48 h. The control sample labeled as "0" refers to untreated control. Loading control: β-Actin. Bands were quantified and results are shown as the ratio between the cleaved/full length protein; *$p<0.05$, **$p<0.01$ *vs. control.*

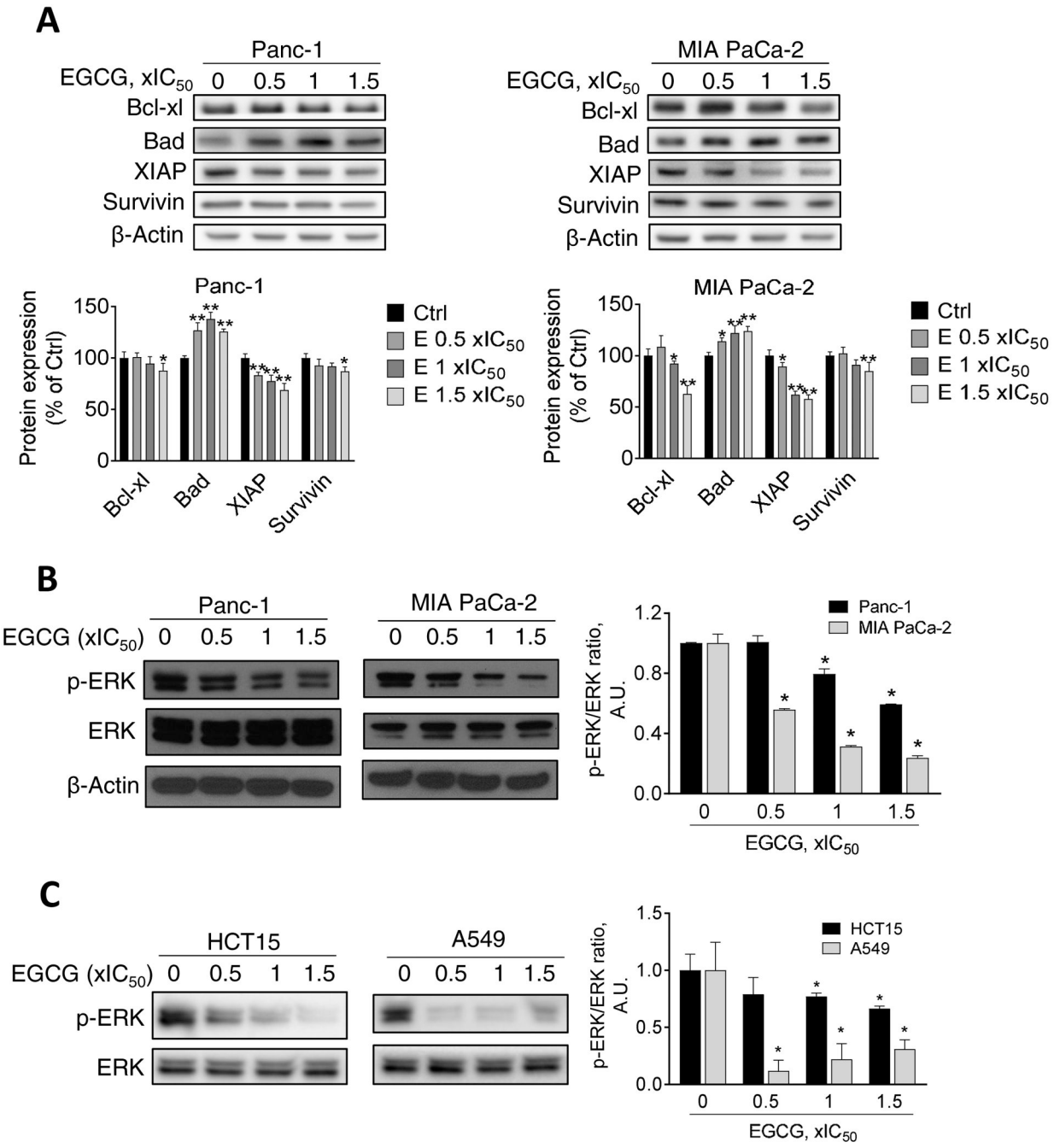


Figure 3. Effect of EGCG on apoptosis-related protein expression and ERK phosphorylation in multiple human cancer cells.

A: EGCG modulates the Bcl-2 family and XIAP family protein expression. Immunoblots for Bcl-xL, Bad, XIAP, and survivin in total cell protein extracts from Panc-1 and MIA PaCa-2 cells treated with escalating concentrations of EGCG, as indicated, for 24 h. Loading control: β-Actin. Bands were quantified and results are expressed as percentage of control. **p<0.05, **p<0.01 vs. control*. **B:** EGCG reduces ERK phosphorylation in Panc-1 and MIA PaCa-2 cells, in a concentration-dependent manner. Results are expressed as the p-ERK/ERK ratio, normalized to control. The sample labeled as "0" refers to an untreated

control. *$p<0.05$, *vs. control*. **C:** EGCG reduces ERK phosphorylation in HCT15 and A549 cells. Immunoblots for p-ERK, and ERK in total cell protein extracts from HCT15 and A549 cells treated with escalating concentrations of EGCG (0.5x, 1x and 1.5x IC_{50}) for 24 h. Results are expressed as the p-ERK/ERK ratio, normalized to control. The sample labeled as "0" refers to an untreated control. *$p<0.05$, *vs. control*.

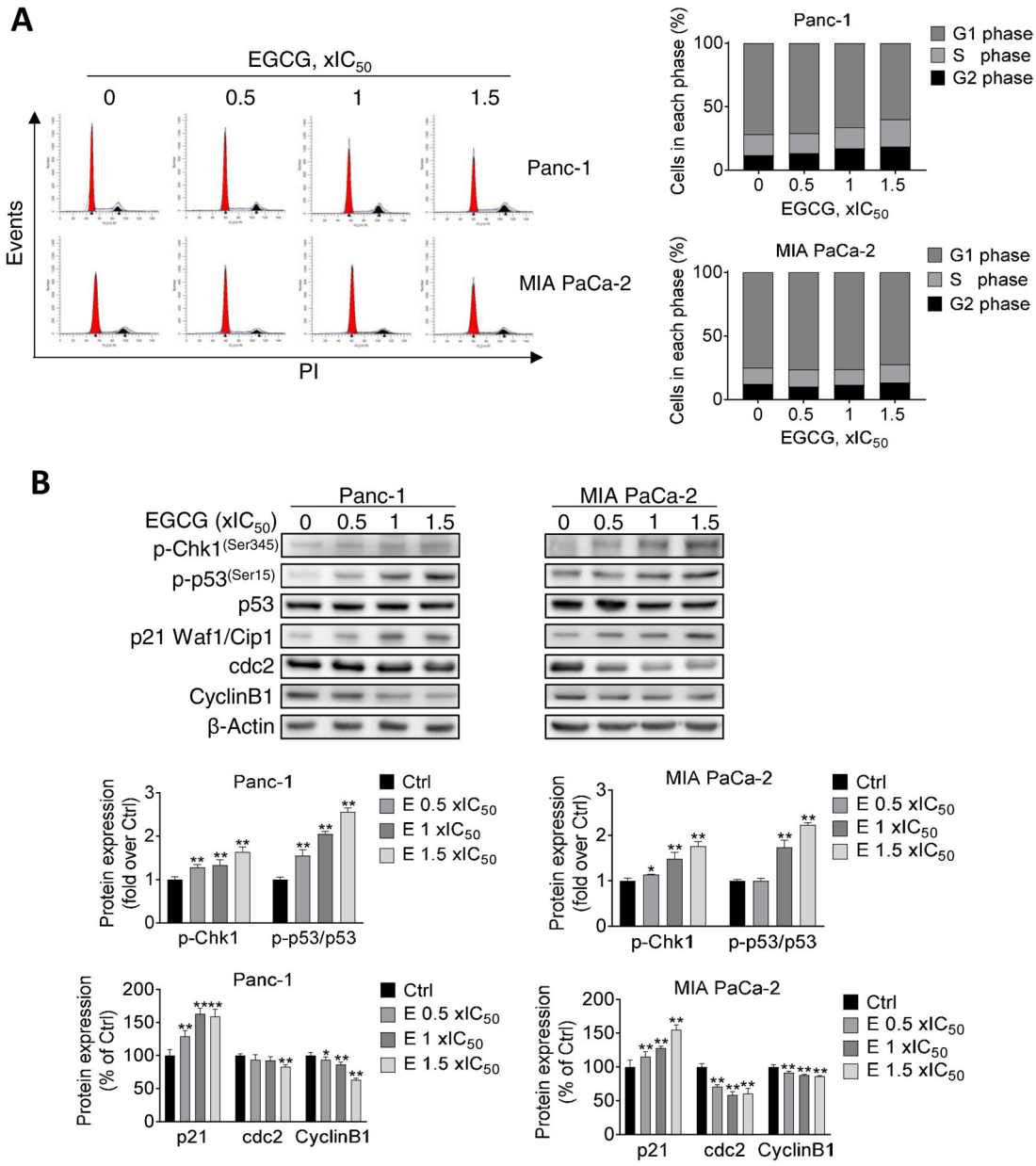


Figure 4. EGCG induces S/G_2 cell cycle arrest in pancreatic cancer cells.

A: Panc-1 and MIA PaCa-2 cells were treated with EGCG for 24 h, as indicated, and the cell cycle phase transition was determined by flow cytometry following PI staining. **B:** EGCG modulated S/G_2 phases arrest protein expression. Immunoblots for phosphorylated Chk1 (p-Chk1), phosphorylated p53 (p-p53), p53, p21, cdc2, and cyclin B1 in total cell protein extracts from Panc-1 and MIA PaCa-2 cells treated with escalating concentrations of EGCG, as indicated, for 24 h. Loading control: β-Actin. Bands were quantified and results are expressed as percentage of control. **p<0.05, **p<0.01 vs. control.*

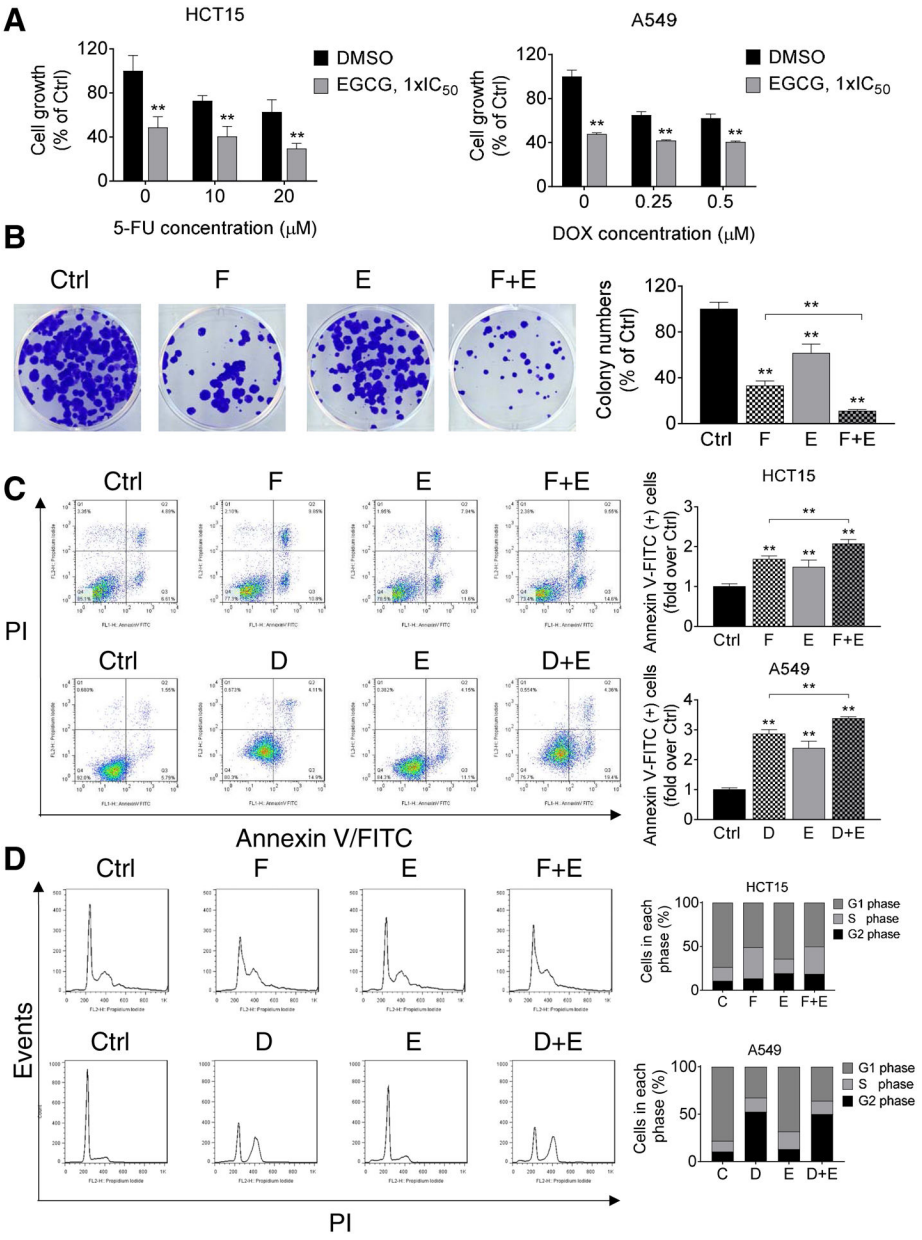


Figure 5. EGCG enhances the cell growth inhibitory effect of 5-FU and Doxorubicin in colon and lung cancer cells.

A: Cell growth was determined in HCT15 and A549 cells following treatment with EGCG ($1xIC_{50}$), 5-FU, doxorubicin (DOX), or combinations of EGCG + 5-FU or EGCG + DOX for 72 h. Results are expressed as a percentage of control. ** *p<0.01 vs. control*. **B:** HCT15 cells were treated with EGCG (E), 5-FU (F), or both (F + E) for 48 h, and the colony formation capacity was determined. Results are expressed as a percentage of control. * *p<0.05*, ** *p<0.01 vs. control*. **C:** HCT15 cells (top) were treated with $1xIC_{50}$ EGCG (E), 10 μM 5-FU (F), or both (F+E) for 48 h, while A549 cells (bottom) were treated with $1xIC_{50}$ EGCG (E), 0.25 μM doxorubicin (D), or both (D+E) for 48 h. The percentage of apoptotic cells were determined by flow cytometry using dual staining (Annexin V and propidium iodide(PI)). The percentages of Annexin V (+) cells was calculated, and results are

expressed as the fold-increase over control. Co-treatment with EGCG (E) further increased the apoptosis rate induced by 5-FU (F) in HCT15 cells and that of doxorubicin (D) in A549 cells, after 48 h. Results are expressed as percentage of control. $*p < 0.05, **p < 0.01$ *vs. control*. **D:** EGCG (E) does not enhance the effect of 5-FU or doxorubicin on the cell cycle. Following treatment with 1xIC_{50} EGCG (E), 10 μM 5-FU (F), 0.25 μM doxorubicin (D), or combinations of these agents for 24 h, cells were stained with PI and the number of cells in each phase of the cell cycle was measured by flow cytometry.

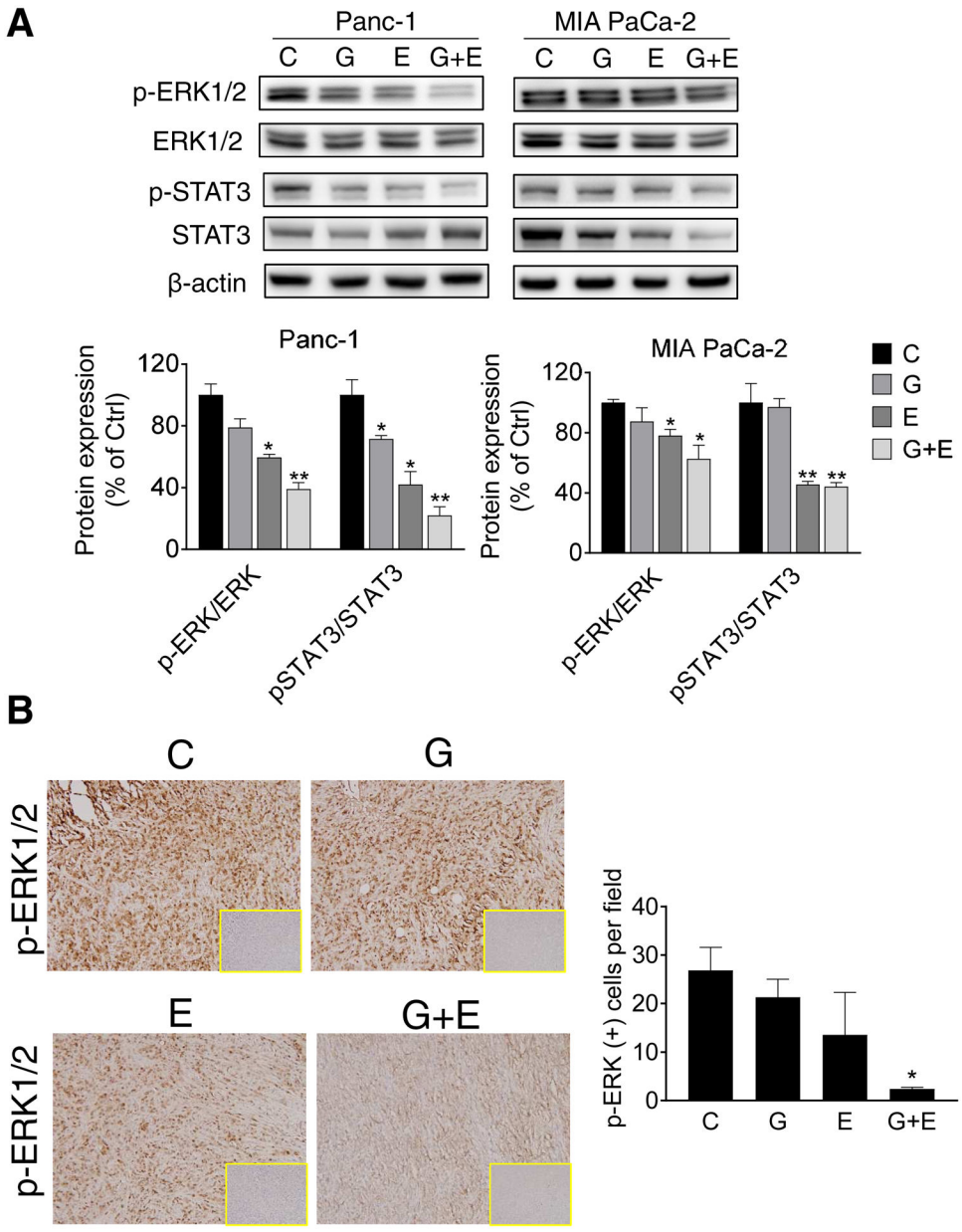


Figure 6: EGCG enhances the effect of gemcitabine on the ERK pathway inhibition in pancreatic cancer.

A: Immunoblots for phosphorylated/total ERK and phosphorylated and total ERK in whole cell protein extracts from Panc-1 and MIA PaCa-2 cells treated with EGCG (E), gemcitabine (G), or both (G + E) for 24 h. Loading control: β-Actin. Bands were quantified and results expressed as the p-ERK/ERK ratio, normalized to control. * *p<0.05*, ** *p<0.01 vs. control.*

B: Immunohistochemistry analysis performed on mouse pancreatic tumor xenograft samples. p-ERK immunostainings were performed on tumor sections and photographs were taken at 20x magnification. Representative images are shown. Results were expressed as percent of p-ERK(+) cells ± SD per 20x field. **Significant compared to control group; p<0.05.*

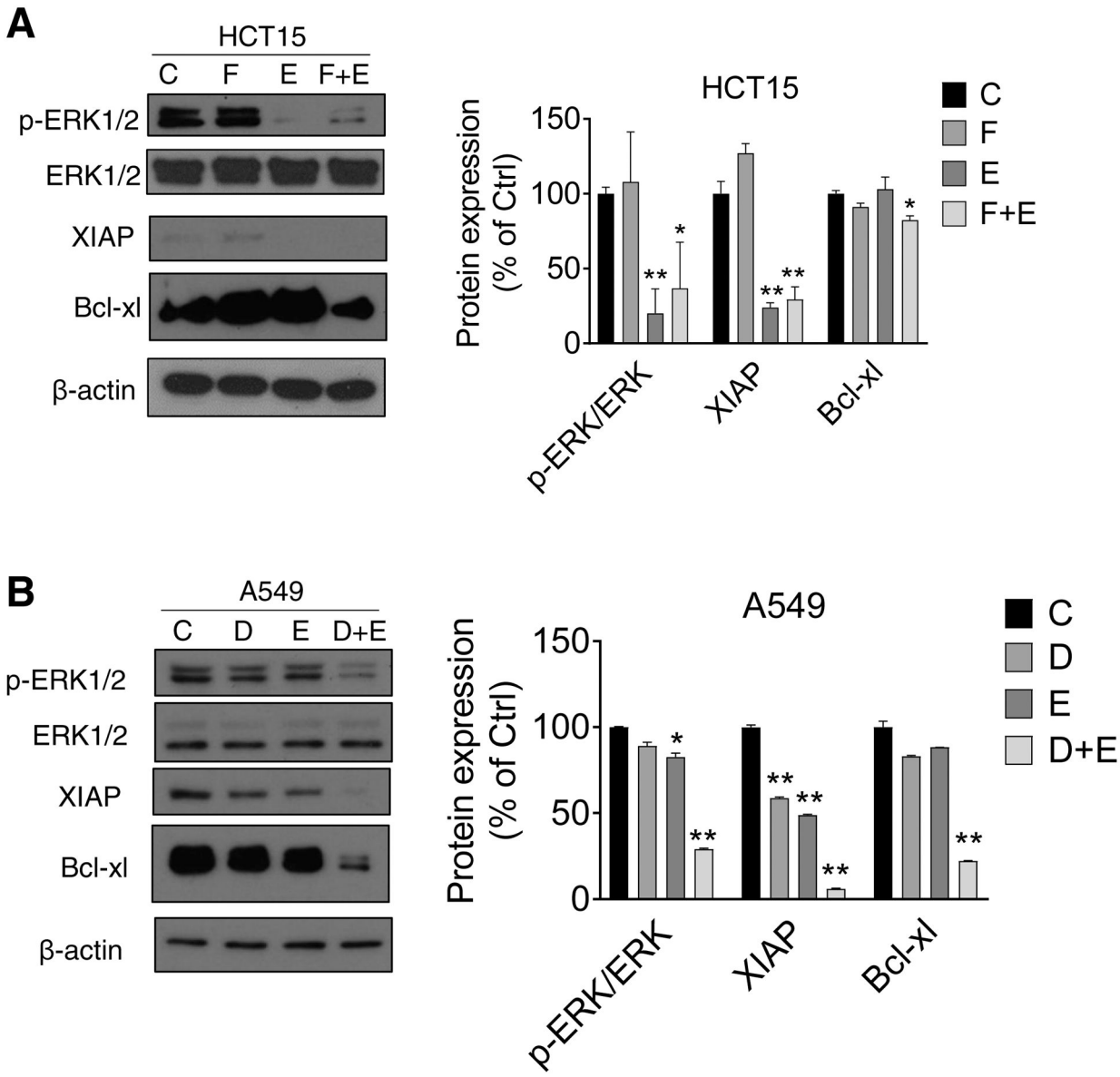


Figure 7: EGCG enhances sensitivity of 5-FU and doxorubicin on ERK inhibition in colon and lung cancer cells.

A: HCT15 cells were treated with 1xIC_{50} EGCG (E), 10 μM 5-FU (F), or both (F+E) for 24 h. Immunoblots for phosphorylated/total ERK, Bcl-xL, and XIAP in whole cell protein extracts from HCT15 cells are shown. Loading control: β-Actin. Bands were quantified and results are expressed as a percentage of control. * *p<0.05*, ** *p<0.01 vs. control*. **B:** A549 cells were treated with 1xIC_{50} EGCG (E), 0.25 μM doxorubicin (D), or both (D+E) for 24 h. Immunoblots for phosphorylated/total ERK, Bcl-xL, and XIAP in whole cell protein extracts from A549 cells are shown. Loading control: β-Actin. Bands were quantified and results are expressed as a percentage of control. * *p<0.05*, ** *p<0.01 vs. control*.