



Modulation of Rad51, ERCC1, and thymidine phosphorylase by emodin result in synergistic cytotoxic effect in combination with capecitabine

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ABSTRACT

Thymidine phosphorylase (TP) is the rate-limiting enzyme for the activation of capecitabine (pro-drug of fluorouracil), and as a useful predictor of tumor response to capecitabine-based chemotherapy. Overexpression of Rad51 and ERCC1 induce resistance to chemotherapeutic agents. Emodin, one of the main bioactive anthraquinone derivatives in the roots and rhizomes of numerous plants, possesses potent antitumor effects. Accordingly, we aimed to explore the molecular mechanism of emodin enhances the capecitabine-induced cytotoxicity through controlling Rad51, ERCC1, and TP expression in human non-small cell lung cancer (NSCLC). The results show that capecitabine increases the phosphorylation of MKK1/2–ERK1/2 and protein levels of Rad51 and ERCC1 through enhancing the protein stability. Depletion of endogenous Rad51 or ERCC1 expression by specific small interfering RNA transfection significantly increases capecitabine-induced cell death and growth inhibition. Emodin enhances the capecitabine-induced cytotoxic effects through ERK1/2 inactivation and decreasing the Rad51 and ERCC1 protein levels induced by capecitabine. Enhancement of ERK1/2 signaling by constitutively active MKK1/2 (MKK1/2-CA) results in increasing Rad51 and ERCC1 protein levels and cell viability in NSCLC cell lines treated with emodin and capecitabine. Interestingly, emodin enhances TP mRNA and protein expression in capecitabine treated NSCLC cell lines, and depletion of the TP expression decreases the cytotoxic effects induced by capecitabine and emodin. We conclude that enhancing the cytotoxicity to capecitabine by emodin is mediated by down-regulation the expression of Rad51 and ERCC1 and up-regulation TP expression.

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1. Introduction

Lung cancer is the most common cause of cancer-related death worldwide. Non-small-cell lung cancer (NSCLC) accounts for 80% of all lung cancer cases [1]. A rational approach to improve the efficacy of single chemotherapeutic agents as second-line therapy of advanced NSCLC is combining agents with different mechanisms of action, non-overlapping toxic effects, and good general tolerance [2,3]. Capecitabine (Xeloda) is a pro-drug of fluorouracil (FU), with the aim of improving tolerability and drug concentrations through its conversion to the active drug in cancer cells through thymidine phosphorylase (TP) [4]. Previous clinical studies have shown that a synergistic antitumor activity by capecitabine plus docetaxel is through up-regulating the expression of TP [5,6]. Furthermore,

higher expression of TP in tumor tissue enhances the conversion of capecitabine to FU in different human cancers [7]. However, the antitumor activity of capecitabine has rarely been evaluated in NSCLC [8]. Therefore, whether the increase of TP expression in NSCLC could provide a novel therapy to enhance the cytotoxic effect of the capecitabine in lung cancer cells needs to be further investigated.

Emodin (1,3,8-trihydroxy-6-methylanthraquinone), an anthraquinone derivative from the root and rhizome of *Rheum palmatum*, a herb widely used in traditional Chinese medicine, has been reported to exhibit anti-cancer effects [9,10]. Emodin can decrease the phosphorylation of ERK1/2 in PC3 prostate cancer cells [11], and antagonize AKT and ERK1/2 signaling to trigger apoptosis in human lung carcinoma cells [12,13]. Moreover, emodin is a strong reactive oxygen species-producing agent [14] and characterized as a genotoxic compound that is able to induce DNA damage [15]. Though the anti-cancer and chemosensitizing effects of emodin has been demonstrated, whether emodin could enhance the

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cytotoxicity induced by capecitabine in NSCLC still needs to be further explored.

Mammalian Rad51 is a central DNA recombinase in the homologous recombination repair (HRR) pathway [16]. Elevated Rad51 levels promote instability of the genome [17] and high expression of Rad51 in NSCLC is associated with an unfavorable prognosis [18,19]. Inhibition of Rad51 expression sensitizes NSCLC cells to chemotherapeutic agents and tyrosine kinase inhibitors [20,21]. Rad51 knockout in mice results in early embryonic lethality [22] and loss of Rad51 function in the chicken DT40 cell line induces chromosome breaks prior to cell death [23]. Deficiencies in Rad51 function can result in genome instability and tumor formation in nude mice [24].

ERCC1 is a leading protein in nucleotide excision repair (NER), and is responsible for recognition of DNA damage and removal of the damaged nucleotides. ERCC1 combines with XPF endonuclease (xeroderma pigmentosum complementation group F, ERCC4) [25,26], and it can act as a heterodimer and function as an endonuclease to catalyze incision on the 5'-side of the damaged DNA [27,28]. In patients with NSCLC, a lower level of ERCC1 expression in cancer cells is correlated with longer survival after treatment with platinum-based chemotherapy when compared with survival of patients with a higher level of ERCC1 in cancer cells [29,30]. However, whether increased ERCC1 is associated with drug resistance to capecitabine is still unclear and remains to be determined.

In this study, we investigated the role of emodin in enhancing the capecitabine-induced cell death in four NSCLC cell lines, and explored the molecular mechanisms involved in the cytotoxic effects. Our studies have shown that emodin has a synergistic effect on capecitabine-induced cytotoxicity via decreasing the expression of Rad51 and ERCC1 and increasing TP expression. The synergistic effect of the combined treatment with capecitabine and emodin on NSCLC provides new and useful information for its application in lung cancer therapy.

2. Materials and methods

2.1. Drugs and reagents

Emodin was purchased from the Sigma Chemical Co. (St. Louis, MO, USA). Capecitabine was obtained from Roche. Cycloheximide and actinomycin D were purchased from Sigma-Aldrich (St. Louis, MO, USA). N-acetyl-Leu-Leu-norleucinal (ALLN), MG132, and U0126 were purchased from Calbiochem-Novabiochem (San Diego, CA, USA). Emodin, capecitabine, actinomycin D, ALLN, MG132, and U0126 were dissolved in dimethyl sulfoxide (DMSO). Cycloheximide was dissolved in Milli-Q-purified water (Millipore, Billerica, MA, USA).

2.2. Cell lines and culture

The human NSCLC cell lines A549, H520, H1975, and H1703 were obtained as previously described [31] and cultured at 37 °C in a humidified atmosphere containing 5% CO₂ in RPMI-1640 complete medium supplemented with sodium bicarbonate (2.2%, w/v), L-glutamine (0.03%, w/v), penicillin (100 units/mL), streptomycin (100 µg/mL), and fetal calf serum (FCS) (10%).

2.3. Western blot analysis

After the different treatments, the cells were rinsed twice with cold PBS and lysed in the whole cell extract buffer (20 mM HEPES [pH 7.6], 75 mM NaCl, 2.5 mM MgCl₂, 0.1 mM EDTA, 0.5% Nonidet P-40, 0.1 mM Na₃VO₄, 50 mM NaF, 1 µg/mL leupeptin, 1 µg/mL aprotinin, 1 µg/mL pepstatin, and 1 mM 4-(2-aminoethyl)benze-

nesulfonyl fluoride) [Sigma]. The cell lysate was rotated at 4 °C for 30 min and then centrifuged at 10,000 rpm for 15 min, after which the precipitates were discarded. The Biocinchonic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA) was used to determine protein concentrations using bovine serum albumin as a standard. Equal amounts of protein from each set of experiments were subjected to western blot analysis. The specific phospho-ERK1/2 (Thr202/Tyr204) and phospho-MKK1/2 (Ser217/Ser221) antibodies was purchased from Cell Signaling (Beverly, MA, USA). Rabbit polyclonal antibodies against Rad51(H-92) (sc-8349), ERCC1 (FL-297) (sc-10785), TP(PGF-44C) (sc-47702), ERK2(K-23) (sc-153), HA(F-7) (sc-7392), and actin(I-19) (sc-1616) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.4. Transfection with small interfering RNA

The sense-strand sequences of siRNA duplexes used for Rad51, ERCC1, TP and scrambled (as a control) were 5'-UGUAGCAUAUGCUCGAGCG-3', 5'-GGAGCUGGCUAAGAUGUGU-3', 5'-AUAGACUCCAGCUUAUCCA-3' and 5'-GCGCGUUUGUAG-GATTCG-3' (Dharmacon Research, Lafayette, CO). Cells were transfected with siRNA duplexes (200 nM) with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) for 24 h.

2.5. Plasmid and transfection

Plasmid transfection of MKK1-CA (a constitutively active form of MKK1, ΔN3/S218E/S222D) and MKK2-CA (a constitutively active form of MKK2, ΔN4/S222E/S226D) was achieved as previously described [31]. Exponentially growing human lung cancer cells (10⁶) were plated for 18 h and the MKK1/2-CA expression vectors were transfected into cells using Lipofectamine (Invitrogen, Carlsbad, CA) before capecitabine and emodin treatment.

2.6. Reverse transcription-PCR

Expression of Rad51, ERCC1, and TP mRNA was evaluated with reverse transcription-PCR (RT-PCR) method. Total RNA was extracted from capecitabine/emodin-treated NSCLC cells with TRIzol reagent (Invitrogen, Carlsbad, CA). cDNA was synthesized by using random hexamers following the Moloney murine leukemia virus reverse transcriptase cDNA synthesis system (Invitrogen, Carlsbad, CA). The final cDNA was used for subsequent PCRs. Rad51 was amplified by using the primers 5'-CTTGGCCCCACAACC-CATTC-3' (forward) and 5'-ATGGCCTTTCCTCACCTCCAC-3' (reverse) in conjunction with a thermal cycling program consisting of 26 cycles of 95 °C for 30 s, 61 °C for 30 s, and 72 °C for 60 s. ERCC1 was amplified by using the primers with the sequence 5'-CCCTGGGAATTTGGCGACGTAA-3' (forward) and 5'-CTCCAGG-TACCGCCAGCTTCC-3' (reverse) in conjunction with a thermal cycling program consisting of 26 cycles of 95 °C for 30 s, 61 °C for 30 s, and 72 °C for 60 s. TP was amplified by using the primers with the sequence 5'-GGCATGGATCTGGAGGAGAC-3' (forward) and 5'-CTCTGACCCACGATACAGCA-3' (reverse) in conjunction with a thermal cycling program consisting of 40 cycles of 95 °C for 30 s, 59 °C for 30 s, and 72 °C for 30 s. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was amplified as an internal control. The GAPDH primers were 5'-CTACATGGTTTACATGTTC-3' (forward) and 5'-GTGAGCTTCCCGTTCAGCTCA-3' (reverse). Expression of GAPDH was used as a control to measure the integrity of the RNA samples.

2.7. Measurement of cell viability

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Tetrazolium dye; Sigma] assays were used to evaluate

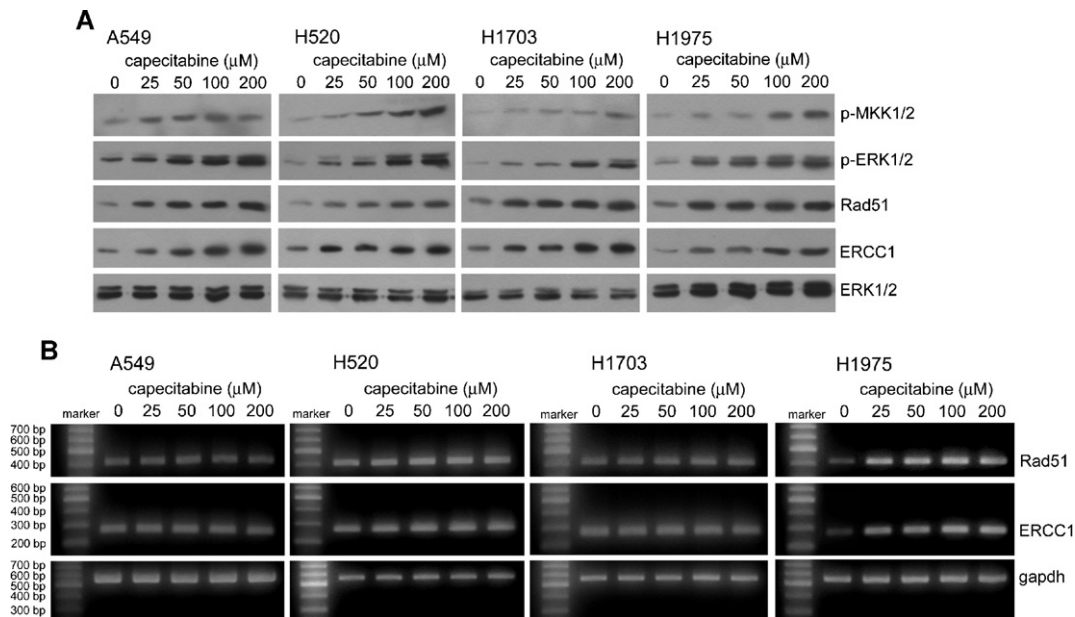


Fig. 1. Capecitabine increased basal phospho-MKK1/2, phospho-ERK1/2, Rad51 and ERCC1 protein levels in human lung cancer cells. (A) Cells (10^6) were cultured in complete medium for 18 h and then exposed to capecitabine (25–200 μM) for 24 h in complete medium. After treatment, cell extracts were examined by western blot for the determination of phospho-MKK1/2, phospho-ERK1/2, Rad51, ERCC1, and ERK1/2 protein expression levels. The western blot shown is one representative of five independent experiments. (B) After treatment, total RNA was isolated and subjected to RT-PCR for Rad51 and ERCC1. The result of RT-PCR experiment is one representative of three independent experiments.

the inhibitory effects of capecitabine and emodin on cell viability as previously described [31]. In brief, cells were seeded on 96-well plates with RPMI containing 10% FCS in a final volume of 0.2 mL, incubated for 18 h, and then treated with the drugs for 24 h. After drug treatment, the MTT solution was added to each well and incubated for 3 h before the medium was removed. DMSO was then added and the plates were shaken for 15 min at room temperature. Cell viability was determined by measuring the absorbance at 562 nm in a microplate reader (Biorad Technologies, Hercules, CA).

2.8. Combination index analysis

The cytotoxicity induced by the combined treatment with capecitabine and emodin was compared with the cytotoxicity

induced by each drug using the combination index (CI), where $CI < 0.9$, $CI = 0.9–1.1$, and $CI > 1.1$ indicate synergistic, additive, and antagonistic effects, respectively [32]. The combination index analysis was performed using CalcuSyn software (Biosoft, Oxford, UK). The CI values at a fraction affected (FA) of 0.75 were averaged for each experiment, and the values were used to calculate the mean between the three independent experiments.

2.9. Trypan blue dye exclusion assay

Cells were treated with capecitabine and/or emodin for 24 h. In each preparation, the cell viability was determined by the trypan blue dye exclusion assay; this dye was excluded by living cells and only penetrated the cell membrane of dead cells. The proportion of

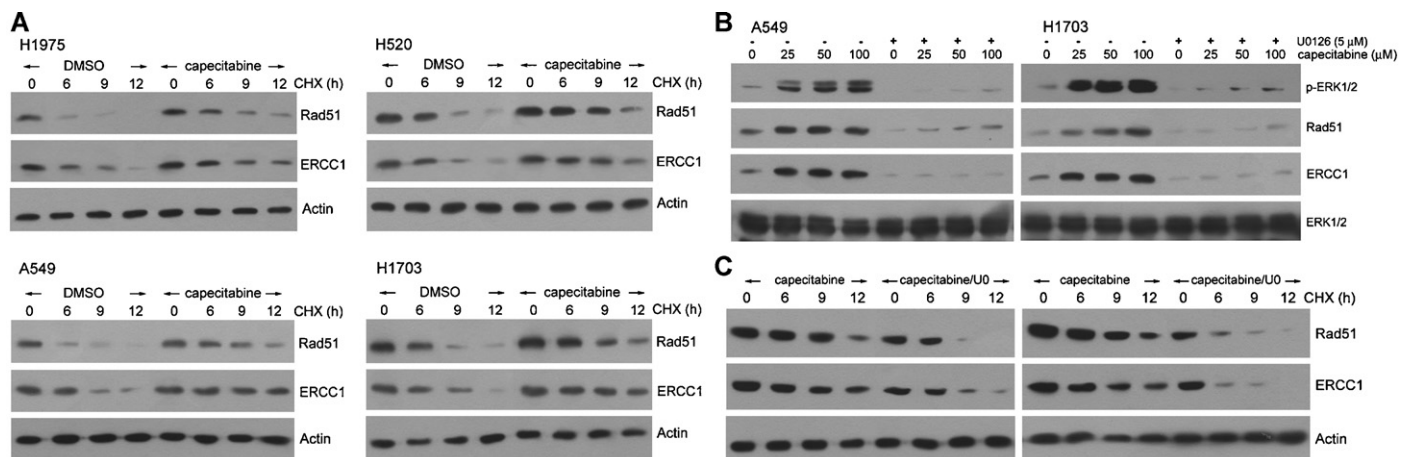


Fig. 2. Capecitabine increases Rad51 and ERCC1 protein stability in ERK1/2 dependent manner. (A) Cells were treated with capecitabine (100 μM) for 12 h followed by treatment with cycloheximide (CHX, 70 μg/mL) for 6–12 h. Whole cell extracts were collected for western blot analysis. (B) MKK1/2 inhibitor U0126 decreases Rad51 protein levels in capecitabine-treated H1703 and A549 cells. Cells were co-treated with capecitabine (25–100 μM) and U0126 (5 μM) for 24 h. (C) Cells were treated with capecitabine (100 μM) and/or U0126 (5 μM) followed by treatment cycloheximide (CHX, 70 μg/mL) for 6–12 h. Whole cell extracts were collected for Western blot analysis for the determination of Rad51 and ERCC1 protein levels. The western blot shown is one representative of four independent experiments.

dead cells was determined by using a hemocytometer to count the number of stained cells.

2.10. Statistical analysis

For each protocol, three or four independent experiments were performed. Results were expressed as the mean \pm standard error of the mean (SEM). Statistical calculations were performed by using SigmaPlot 2000 (Systat Software, San Jose, CA). Differences in measured variables between experimental and control groups were assessed by the unpaired *t*-test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Capecitabine increases phosphorylated protein levels of MKK1/2 and ERK1/2

To evaluate the molecular mechanisms of capecitabine-induced cytotoxicity in different NSCLC cell types, these cell lines were exposed to various concentrations of capecitabine (25–200 μ M) for 24 h. Phosphorylated protein levels of MKK1/2 and ERK1/2 were determined by western blot analysis. In Fig. 1A, capecitabine dose-dependently increased basal protein levels of phosphorylated MKK1/2 and ERK1/2, whereas there was no change in the endogenous protein levels of ERK1/2.

Table 1

Protein half-life (h) of Rad51 and ERCC1 in capecitabine and U0126 co-exposed NSCLC cell lines.

Cell line	Rad51 half-life (h)			ERCC1 half-life (h)		
	DMSO	Cap	U0/Cap	DMSO	Cap	U0/Cap
H1975	5.6	10.2	N/A	9.0	12.0	N/A
A549	6.0	12.0	7.6	9.7	>12.0	8.6
H520	8.4	>12.0	N/A	12.0	>12.0	N/A
H1703	8.0	10.4	6.0	10.0	>12.0	5.4

N/A: non-detected; Cap: capecitabine; U0: U0126.

3.2. Rad51 and ERCC1 protein levels are increased under capecitabine exposure

In this study, we wanted to know whether Rad51 and ERCC1 play a protective role in capecitabine-induced cytotoxic effects in NSCLC cells. Protein levels of Rad51 and ERCC1 were determined by western blot analysis. Capecitabine significantly and dose-dependently increased the basal protein levels of Rad51 and ERCC1 (Fig. 1A). To elucidate the mechanisms responsible for the elevated Rad51 and ERCC1 expression observed in NSCLC cell lines, we further confirmed the expression of Rad51 and ERCC1 mRNA by RT-PCR. As shown in Fig. 1B, cellular Rad51 and ERCC1 mRNA levels were not affected by capecitabine in A549, H1703, and H520 cells. However, capecitabine increased the Rad51 and ERCC1 mRNA expression in H1975 cells.

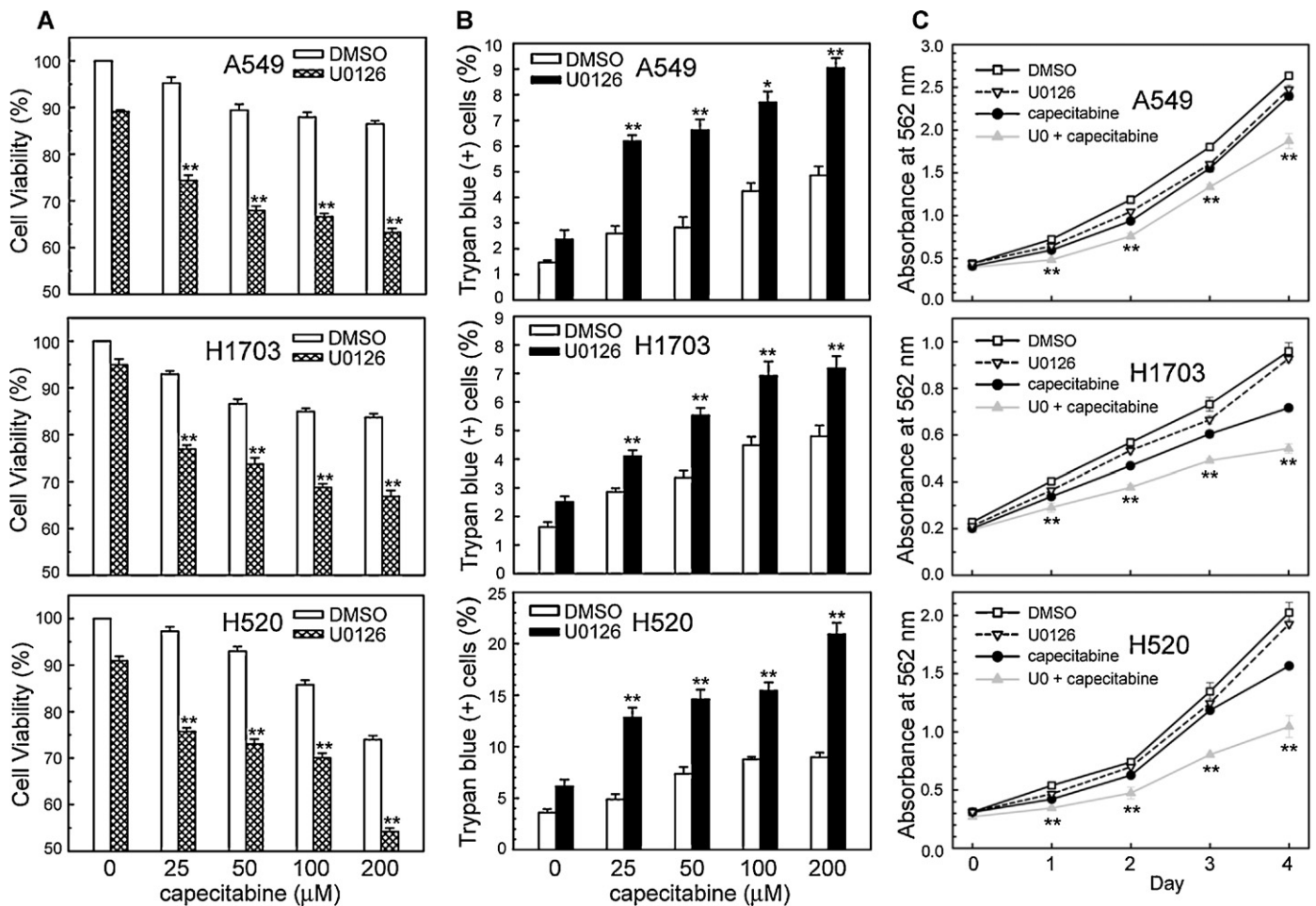


Fig. 3. The influence of the MKK1/2–ERK1/2 signaling pathway on cell viability in NSCLC cells treated with capecitabine. (A and B) Cells were treated with different concentration of capecitabine and U0126 (5 μ M) for 24 h. Viable cells were evaluated by the MTT and trypan blue exclusion assays. The results (mean \pm SEM) were obtained from four independent experiments. (C) Cells were treated with capecitabine (100 μ M) with or without U0126 (5 μ M) for 1–4 days. The viable cells were determined by the MTT assay. The results (mean \pm SEM) were from four independent experiments. Asterisks (* and **) denote $P < 0.05$ and $P < 0.01$, respectively, using the Student's *t*-test for the comparisons between cells treated with or without U0126.

3.3. Capecitabine enhances the protein stability of Rad51 and ERCC1

We next examined the mechanism that was involved in Rad51 and ERCC1 protein expression induced by capecitabine. The protein synthesis inhibitor, cycloheximide, was added to culture media to evaluate the protein degradation rate of Rad51 and ERCC1. We found that when these four different NSCLC cell lines were treated with capecitabine, the protein degradation rate of Rad51 and ERCC1 markedly decreased compared to that treated with DMSO only (Fig. 2A; Table 1). These results suggest that capecitabine-enhanced expression of Rad51 and ERCC1 is mainly due to increasing the protein stability of Rad51 and ERCC1.

3.4. ERK1/2 activation is correlated with enhancement of Rad51 and ERCC1 proteins

Next, we determined whether the ERK1/2 signaling pathway was involved in the regulation of Rad51 and ERCC1 protein expression in capecitabine-exposed NSCLC cells. The A549 and H1703 cell lines were treated with MKK1/2 inhibitor U0126 and various concentrations of the capecitabine for 24 h. As shown in Fig. 2B, capecitabine enhanced ERK1/2 phosphorylation, and the Rad51 and ERCC1 protein levels were significantly decreased by U0126 treatment. Moreover, when compared with capecitabine treatment alone, the Rad51 and ERCC1 protein were less stable

when these cells were treated with capecitabine and U0126, and these results were confirmed by cycloheximide chase analysis (Fig. 2C and Table 1). Taken together, these data show that the activation of MKK1/2–ERK1/2 signaling pathway correlates with the induction of Rad51 and ERCC1 proteins in capecitabine-treated NSCLC cells.

3.5. MKK1/2 inhibitor U0126 enhances cytotoxicity induced by capecitabine in NSCLC cells

To evaluate the effects of ERK1/2 inactivation on cytotoxicity induced by capecitabine in NSCLC cells, we examined the influence of U0126 on cell viability inhibited by capecitabine in each cell line. Cell viability was assessed by MTT assay and trypan blue exclusion assay. In Fig. 3A and B, cell viability of NSCLC gradually decreased as the concentrations of capecitabine increased from 25 to 200 μM . Furthermore, greater cell death was observed in the presence of a fixed concentration of U0126 (5 μM) and capecitabine. We also determined the anti-proliferation effect of NSCLC cells treated with capecitabine for 1–4 days by MTT assay, and we found that capecitabine could inhibit the cells growth of NSCLC, and adding U0126 to these cells could significantly enhance this growth suppression in NSCLC (Fig. 3C). Therefore, ERK1/2 activation is associated with anti-cytotoxic effect in capecitabine-treated lung cancer cells.

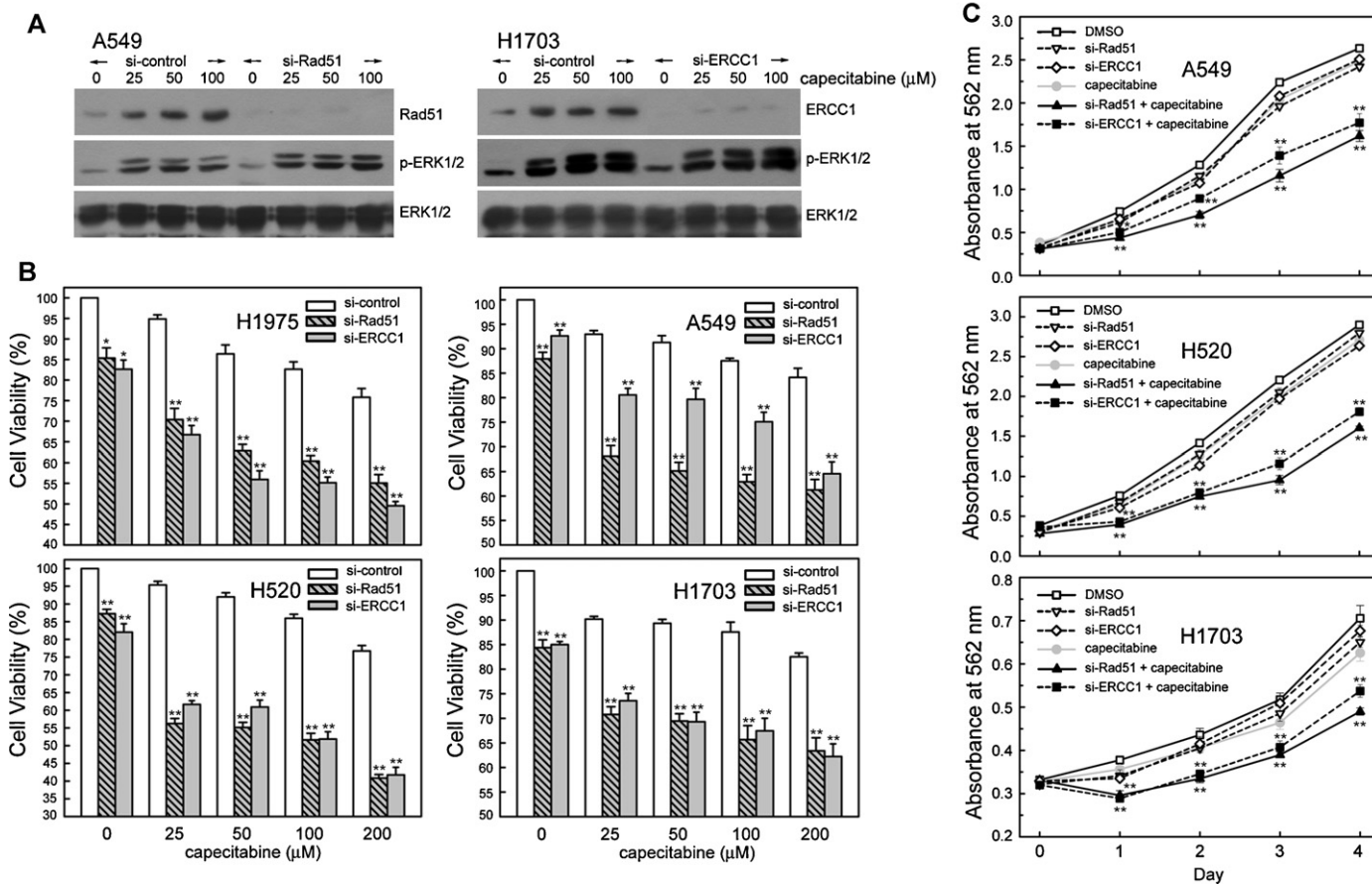


Fig. 4. Knockdown of Rad51 and ERCC1 expression by siRNA transfection enhances cell death induced by capecitabine. (A) A549 and H1703 cells were transfected with siRNA duplexes (200 nM) specific to Rad51, ERCC1, or scrambled control in complete medium for 24 h prior to treatment with capecitabine (25–200 μM) for 24 h in complete medium. Whole cell extracts were collected for western blot analysis using specific antibodies against Rad51, ERCC1, and phospho-ERK1/2. The western blot shown is one representative of four independent experiments. (B) After treatment as above, cytotoxicity was determined by the MTT assay. The results (mean \pm SEM) were from four independent experiments. (C) The si-Rad51, si-ERCC1 or si-scrambled RNA transfected the cells were treated with or without capecitabine (50 μM) for 1–4 days. The viable cells were determined by the MTT assay. The results (mean \pm SEM) were from four independent experiments. Asterisks (**) denote $P < 0.01$, respectively, using the Student's *t*-test for the comparison between cells treated with capecitabine in si-Rad51 RNA, si-ERCC1 RNA or si-scrambled RNA transfected cells.

3.6. Knockdown of Rad51 or ERCC1 sensitizes NSCLC cells to cytotoxicity induced by capecitabine

To determine the roles of Rad51 or ERCC1 on the cytotoxic effects of capecitabine in NSCLC cells, Rad51 or ERCC1 was knocked down using specific siRNA duplexes. As shown in Fig. 4A, transfection of si-Rad51 or si-ERCC1 RNA duplex suppressed Rad51 or ERCC1 protein levels without affecting ERK1/2 phosphorylation in capecitabine-treated A549 or H1703 cells. The cytotoxicity induced by capecitabine and si-Rad51 or si-ERCC1 RNA transfection in NSCLC cell lines was determined by MTT assay. Interestingly, siRNA transfection to knock down the expression of Rad51 or ERCC1 in lung cancer cells was found to enhance the cytotoxicity and cell growth inhibition induced by capecitabine as compared to that by si-scramble RNA transfection (Fig. 4B and C). The data collectively suggest that the expressions of Rad51 and ERCC1 induced by capecitabine are mediated by ERK1/2 signaling activation, which protects NSCLC cells against capecitabine-induced cytotoxicity.

3.7. Emodin decreases the levels of phosphorylated ERK1/2, Rad51 and ERCC1 protein levels induced by capecitabine

Our previous studies showed that emodin could downregulate the protein and mRNA of Rad51 and ERCC1 [33]. We then examined the combined effects of capecitabine and emodin on the expression of Rad51 and ERCC1, and compared it with each separate treatment. As shown in Fig. 5A, emodin alone or combined with capecitabine yielded substantial downregulation of Rad51 and ERCC1 in H1975 and A549 cells. Moreover, capecitabine-induced phosphorylation of ERK1/2 was diminished by emodin (Fig. 5A). We also observed the inhibition of mRNA expressions of Rad51 and ERCC1 in NSCLC

treated with capecitabine and emodin which was shown by RT-PCR analyses (Fig. 5B). Next, we examined the possible mechanisms for posttranscriptional regulation of ERCC1 and Rad51 mRNA. To evaluate the mRNA stability of Rad51 and ERCC1 in capecitabine and emodin-treated NSCLC cells, we treated cells with actinomycin D to block *de novo* RNA synthesis, and then measured the levels of the existing Rad51 and ERCC1 mRNA by RT-PCR at 3, 6, and 9 h after treatment. As shown in Fig. 5C, our results revealed that emodin could decrease the mRNA stability of Rad51 and ERCC1 in NSCLC treated with capecitabine.

3.8. Emodin upregulates TP protein and mRNA expression

Previous studies have indicated that capecitabine plus docetaxel induced a synergistic antitumor effect through increasing TP expression [5,6]. In advanced NSCLC, TP expression was associated with tumor response to capecitabine [34]. Notably, emodin could enhance the basal level of TP mRNA in NSCLC cells (Fig. 5B and D). Moreover, emodin alone or combination with capecitabine could increase cellular TP protein expression when compared with control groups, but the phenomenon was not seen in NSCLC treated with capecitabine alone (Fig. 5E). In addition, consistent with our previous studies, emodin alone could downregulate the Rad51 and ERCC1 mRNA and protein expressions and the phosphorylation of ERK1/2 in NSCLC cells (Fig. 5D and E).

3.9. Rad51 and ERCC1 protein instability is associated with 26S proteasome-mediated proteolysis in capecitabine and emodin co-treated NSCLC cells

To investigate whether the changes in the expression of Rad51 and ERCC1 protein induced by capecitabine and emodin were also

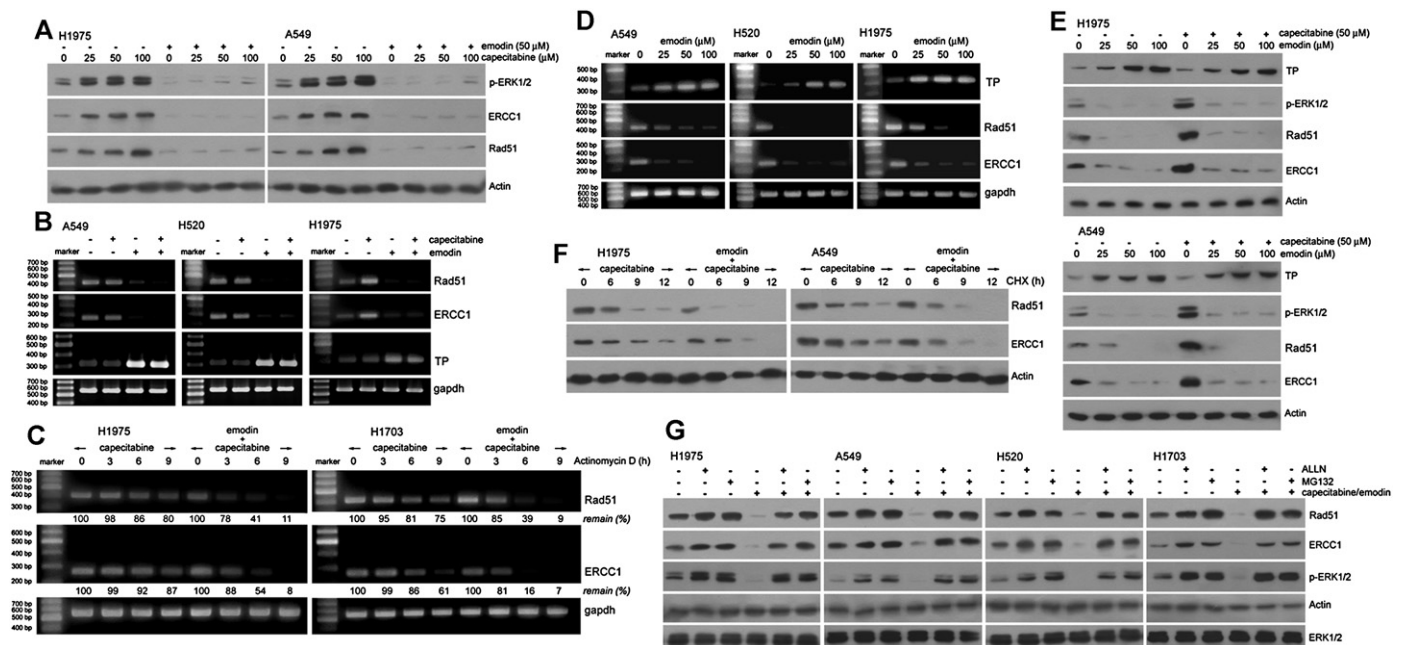


Fig. 5. Emodin decreases capecitabine-induced phospho-ERK1/2, Rad51 and ERCC1 protein levels in human lung cancer cells. (A) H1975 and A549 cells were exposed to capecitabine (25–100 μM) and emodin (50 μM) for 24 h in complete medium. After treatment, cell extracts were examined by western blot for the determination of phospho-ERK1/2, Rad51, ERCC1, and ERK1/2 protein expression levels. The western blot shown is one representative of four independent experiments. (B) Cells were treated with capecitabine (100 μM) and/or emodin (50 μM) for 24 h, total RNA was isolated and subjected to RT-PCR for Rad51 and ERCC1. (C) Cells were exposed to capecitabine (100 μM) alone or in combination with emodin (50 μM) for 9 h, followed by the addition of actinomycin D (2 μg/mL) for 3–9 h. After treatment, total RNA was isolated and subjected to RT-PCR for Rad51 and ERCC1. (D) Cells were exposed to different concentrations of emodin (25–100 μM) for 24 h. After treatment, total RNA was isolated and subjected to RT-PCR. The result of RT-PCR experiment is one representative of three independent experiments. (E) Cells were exposed to emodin (25–100 μM) and capecitabine (50 μM) for 24 h in complete medium. After treatment, cell extracts were examined by western blot for the determination of TP, phospho-ERK1/2, Rad51 and ERCC1 protein expression levels. (F) Cells were treated with capecitabine (100 μM) and/or emodin (50 μM) for 12 h followed by treatment with cycloheximide (CHX, 70 μg/mL) for 6–12 h. (G) Capecitabine (50 μM) and emodin (50 μM) were added to NSCLC cells for 18 h. Cells were then treated with MG132 (10 μM) or ALLN (10 μM) for 6 h. Whole cell extracts were collected for western blot analysis. The western blot shown is one representative of three independent experiments.

regulated at the posttranslational level, cycloheximide was added in cultured media with capecitabine and/or emodin for 6–12 h. In Fig. 5F, emodin treatment significantly enhanced Rad51 and ERCC1 degradation after treatment with capecitabine and cycloheximide. Therefore, Rad51 and ERCC1 were less stable after treatment with emodin and capecitabine when compared with capecitabine alone. Taken together, these results suggested that the downregulation of Rad51 or ERCC1 protein expression by co-treatment with capecitabine and emodin was due to the decrease in its mRNA and protein stability.

To investigate whether the 26S proteasome was involved in the emodin-induced degradation of Rad51 and ERCC1 proteins, the 26S proteasome inhibitors MG132 or ALLN was co-added with capecitabine and emodin to NSCLC cells. As shown in Fig. 5G, both MG132 and ALLN restored the Rad51 and ERCC1 protein levels that had been decreased by capecitabine and emodin. Moreover, MG132 or ALLN could increase protein levels of phosphorylated ERK1/2 in these four NSCLC cell lines, whereas no differences in endogenous unphosphorylated-ERK1/2 were observed. Consistent with our previous studies, MG132 or ALLN could stabilize Rad51 and ERCC1 via ERK1/2 activation. Taken together, these results revealed that 26S proteasome-mediated proteolysis is the

mechanism of Rad51 and ERCC1 protein instability enhanced by emodin and capecitabine in NSCLC cells.

3.10. Emodin promotes the cytotoxic effect induced by capecitabine in NSCLC cells

Taxanes or mitomycin C (MMC), as a TP-inductor, combinations with capecitabine could increase the cytotoxic potential of capecitabine [35]. Rad51 and ERCC1 play protecting role in chemotherapeutic agents induced cytotoxicity in NSCLC [36,37]. The above results have shown that emodin could enhance the TP expression and downregulate repair protein Rad51 and ERCC1 expression in capecitabine-exposed NSCLC cells. Therefore, we proposed that emodin could enhance cytotoxic effects of capecitabine. In Fig. 6, NSCLC cells were treated with capecitabine and/or emodin for 24 h, the viable cells were evaluated by the MTT and trypan blue exclusion assays. Capecitabine alone induced only limited cell death of NSCLC. However, these four NSCLC cell lines exhibited a significant decrease in cell viability when the cells were co-treated with capecitabine and emodin (Fig. 6A). Similar phenomena were observed when cell death was measured by trypan blue exclusion assay (Fig. 6B). The combined effects of the

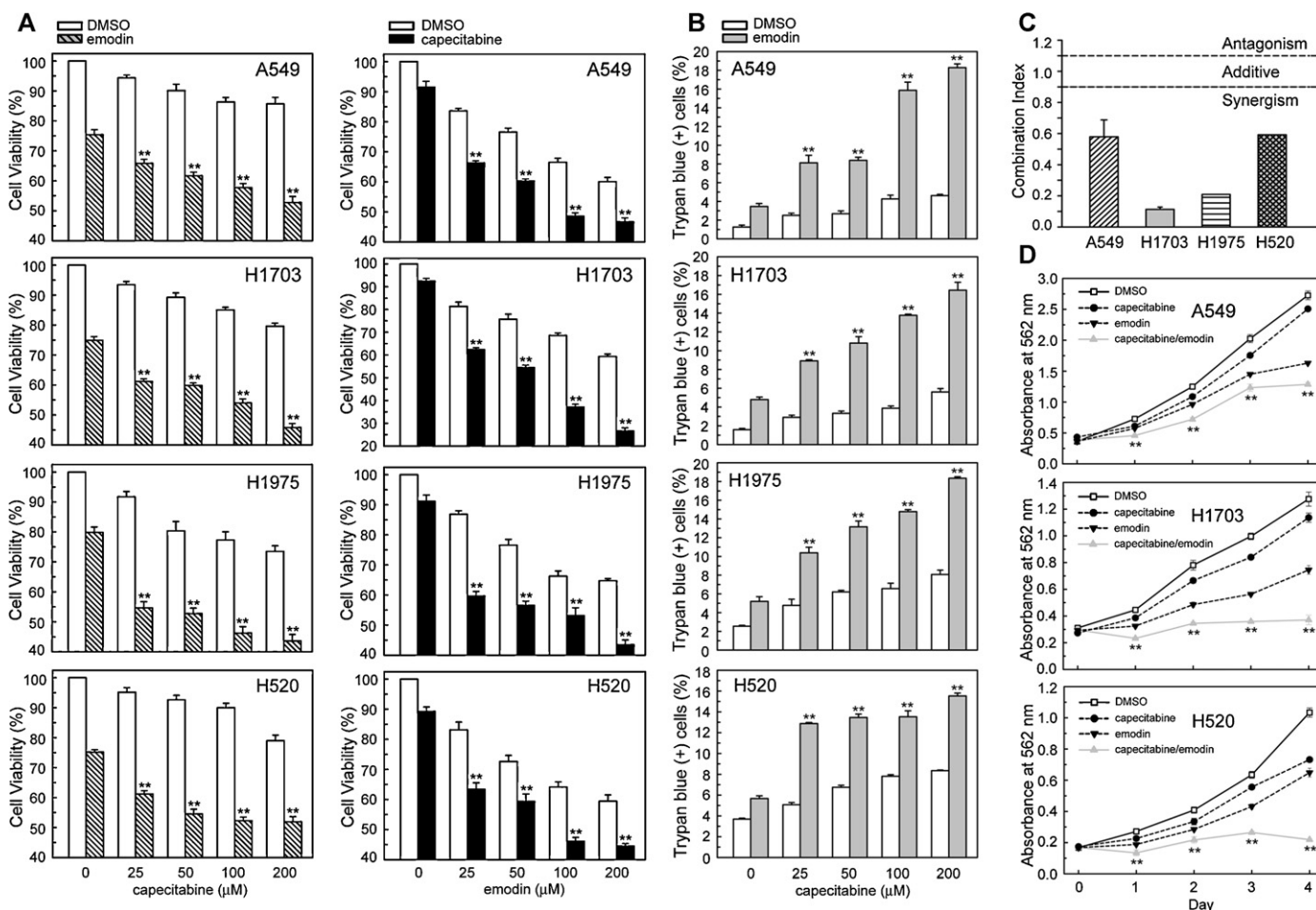


Fig. 6. Emodin cotreatment synergistically enhances capecitabine induced cytotoxicity. (A) Left panel, cells were treated with various concentrations of capecitabine (25–200 μM) and emodin (50 μM) for 24 h. Right panel, cells were treated with emodin (25–200 μM) and capecitabine (50 μM) for 24 h. Cytotoxicity was determined by the MTT assay. The results (mean \pm SEM) were from three independent experiments. (B) At the end of treatment as in (A), unattached and attached cells were collected and stained with trypan blue dye. The numbers of stained cells (dead) were manually counted. (Columns) Percentage of trypan blue-positive cells, representing the population of dead cells; (bar) SE from three independent experiments. Asterisks (* and **) denote $P < 0.05$ and $P < 0.01$, respectively, using the Student's *t*-test for the comparison between cells treated with capecitabine/emodin alone and cells co-treated with capecitabine–emodin. (C) The mean CI values for the capecitabine–emodin combination treatment in NSCLC cells. CI values were averaged for each experiment and the values were used to calculate the mean between experiments. Points and columns, mean values obtained from four independent experiments; bars, standard error (S.E.). (D) Cells were treated with capecitabine (50 μM) and/or emodin (50 μM) for 1–4 days, the viable cells were determined by MTT assay. The results (mean \pm SEM) were from three independent experiments. Asterisks (***) denote $P < 0.01$ using the Student's *t*-test for comparison between cells treated with capecitabine or emodin alone and those co-treated with capecitabine–emodin.

drugs were analyzed as described in Section 2. When the combination index (CI) values significantly <1 indicate synergy; values close to 1 indicate an additive effect; and values significantly >1 indicate an antagonistic effect of the two agents. As seen in Fig. 6C, the CI values for A549, H1703, H1975, and H520 cells were 0.5790, 0.1131, 0.2942, and 0.4671, respectively, supporting a synergistic cytotoxic effects induced by capecitabine and emodin. We also determined the anti-proliferation effect of emodin and capecitabine by MTT assay, and found that emodin could enhance capecitabine-induced cell growth suppression in NSCLC cells (Fig. 6D).

3.11. Influence of ERK1/2 inactivation on the regulation of Rad51 and ERCC1 protein levels under capecitabine and emodin co-treatment

Our results have demonstrated that capecitabine-induced expression of Rad51 and ERCC1 is correlated with ERK1/2 activation, we next tested whether emodin-dependent ERK inactivation is essential for synergistic cytotoxicity induced by capecitabine and emodin. The NSCLC cells were transfected with MKK1/2-CA (constitutively active MKK1/2) vectors, and then treated with emodin and capecitabine for 24 h. Consistent with our

previous studies, as shown in Fig. 7A, phosphorylation levels of ERK1/2 were higher in MKK1-CA or MKK2-CA-transfected cells versus pcDNA3-transfected A549 cells. MKK1/2-CA transfection also could rescue the phosphorylated ERK1/2, Rad51 and ERCC1 protein levels decreased by treatment with emodin and capecitabine (Fig. 7A). These findings indicate that the ERK1/2 inactivation is involved in the downregulation of the expression of Rad51 and ERCC1.

Furthermore, transfection with the MKK1-CA vector increased Rad51 and ERCC1 protein stability in H1703 and A549 cells co-treated with capecitabine and emodin as compared to the protein stability in pcDNA3 transfected cells, which were examined by cycloheximide chase analysis (Fig. 7B). Taken together, emodin decreased capecitabine-induced Rad51 and ERCC1 protein instability were through ERK1/2 inactivation.

3.12. Capecitabine and emodin-induced cytotoxicity is abrogated in MKK1/2-CA overexpressed NSCLC cells

To evaluate the effects of ERK1/2 activation on cell survival suppressed by capecitabine and emodin, the lung cancer cells lines were transfected with MKK1/2-CA vectors, followed by treatment

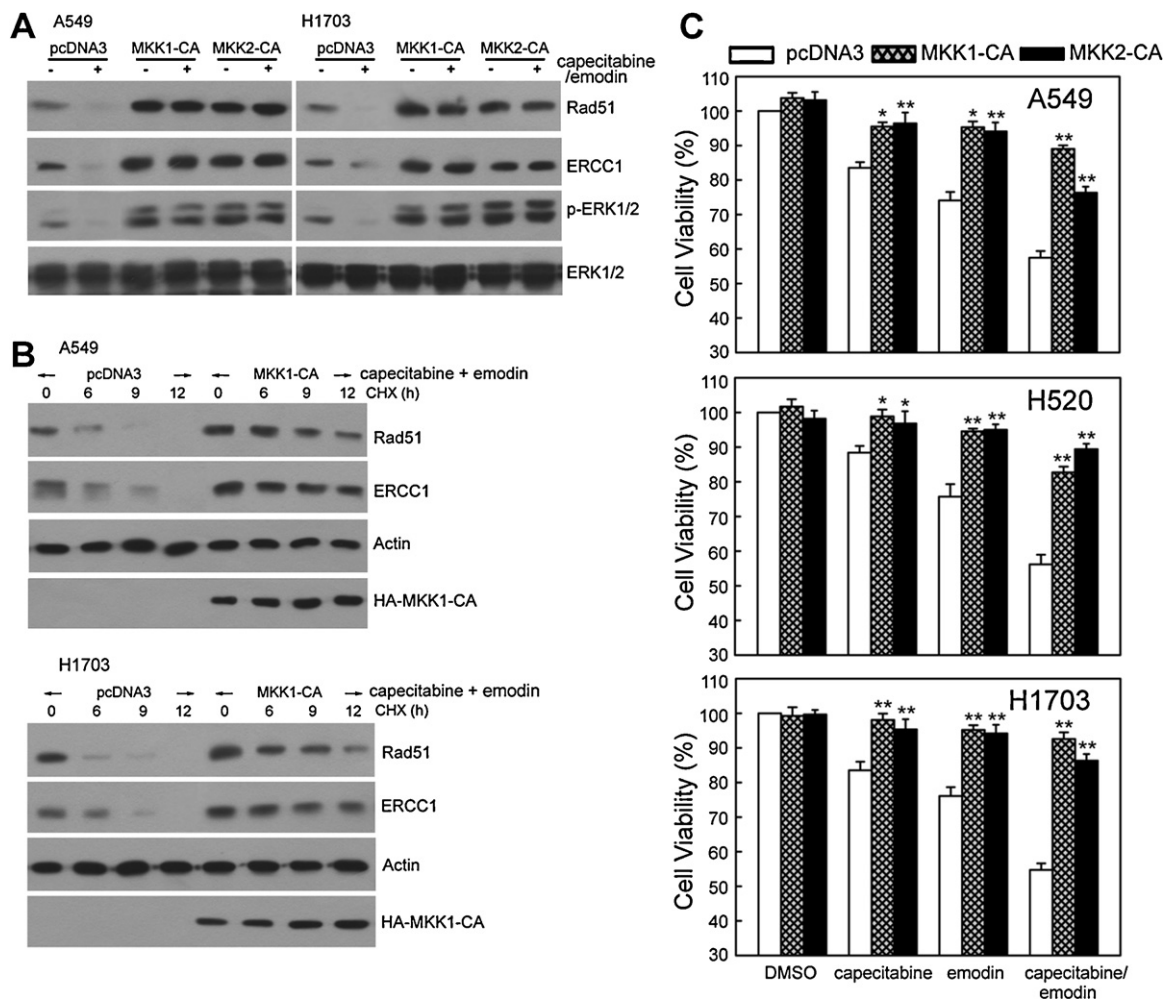


Fig. 7. Overexpression of MKK1/2-CA restores capecitabine and emodin-suppressed ERK1/2 activation, Rad51 and ERCC1 expression and cell viability. (A) MKK1/2-CA expression vectors were transfected into A549 and H1703 cells using Lipofectamine. After expression for 24 h, the cells were treated with capecitabine (100 μ M) and emodin (50 μ M) as described in the legend for Fig. 5. (B) MKK1/2-CA expression vectors transfected A549 and H1703 cells were treated with capecitabine (100 μ M) and emodin (50 μ M) for 12 h, followed by the addition of cycloheximide for 6–12 h. Western blot analysis was performed using specific antibodies against Rad51 and ERCC1. The western blot shown is one representative of four independent experiments. (C) Cells were transfected with MKK1/2-CA vectors for 24 h as above. Capecitabine (100 μ M) and/or emodin (50 μ M) were then added and the cells were incubated for 24 h. The cytotoxicity affected by MKK1/2-CA vector transfection was determined by the MTT assay. The results (mean \pm SEM) were obtained from four independent experiments. Asterisks (* and **) denote $P < 0.05$ and $P < 0.01$, respectively, using the Student's *t*-test for the comparisons between cells transfected with the MKK1/2-CA or pcDNA3 vectors.

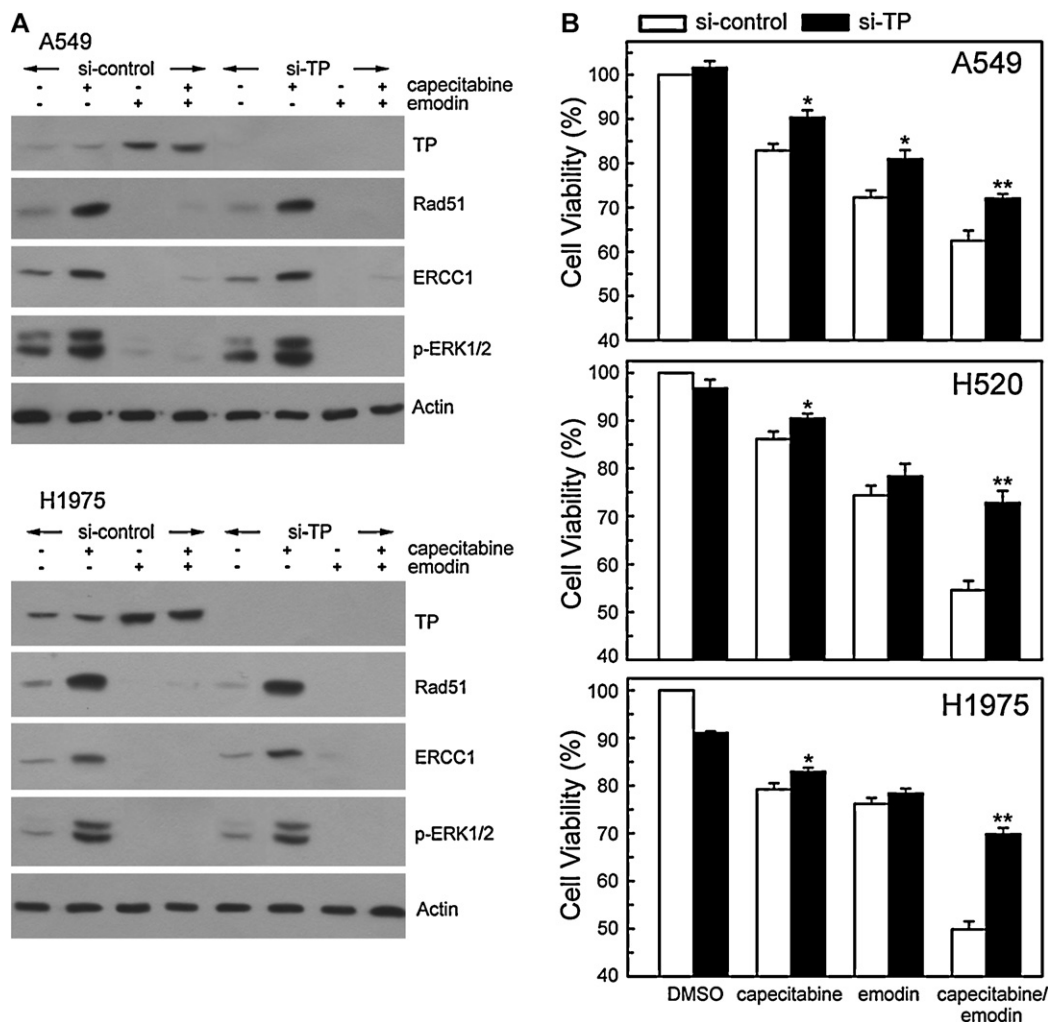


Fig. 8. Knockdown of TP expression by siRNA transfection rescue cell viability decreased by capecitabine and emodin. (A) A549 or H1975 cells were transfected with si-TP RNA duplexes (200 nM) or scrambled control in complete medium for 24 h prior to treatment with capecitabine (100 μ M) and emodin (50 μ M) for 24 h in complete medium, whole cell extracts were collected for western blot analysis using specific antibodies against TP. The western blot shown is one representative of three independent experiments. (B) Cytotoxicity was determined by the MTT assay. The results (mean \pm SEM) were from four independent experiments. Asterisks (**) denote $P < 0.01$, respectively, using the Student's *t*-test for the comparison between cells treated with capecitabine/emodin in si-TP RNA or si-scrambled RNA transfected cells.

with capecitabine and/or emodin. Cell viability was assessed by MTT assay. Transfection with MKK1/2-CA vectors enhanced cell survival suppressed by capecitabine and/or emodin in four different NSCLC cell lines (Fig. 7C). These results suggest emodin could enhance cytotoxicity in capecitabine-exposed NSCLC cells through the inhibition of the MKK1/2-ERK1/2 pathway.

3.13. Knockdown TP expression abrogates the emodin-enhanced cytotoxicity induced by capecitabine in NSCLC cells

To confirm that TP induction by emodin was also associated with enhanced cytotoxicity by capecitabine, we silenced the TP expression using siRNA transfection. As shown in Fig. 8A, si-TP RNA transfection could knock down the TP protein levels which were induced by treatment with emodin. However, knockdown TP expression did not affect the expression of Rad51, ERCC1, or phospho-ERK1/2 in emodin and/or capecitabine treated lung cancer cells (Fig. 8A). Interestingly, emodin significantly increased the cytotoxicity of capecitabine, which was prominently abolished by si-TP RNA transfection in NSCLC cell lines (Fig. 8B). For example, compare with si-control RNA transfected lung cancer cells, cell viability was increased by $\sim 20\%$ in emodin and capecitabine co-treated H520 cells when transfected with the TP siRNA, suggesting

that the increased TP expression by emodin could enhance the cytotoxicity of capecitabine.

4. Discussion

Our prior findings have shown that Rad51 and ERCC1 are down-regulated in NSCLC cells treated with emodin [33], leading us to propose the hypothesis that NSCLC cells would therefore be more sensitive to capecitabine when combined treatment with emodin. In this study, emodin could increase the cytotoxic effects of capecitabine in NSCLC cell lines through suppression of Rad51 and ERCC1 expression and enhancement of TP expression. Moreover, MKK1/2-ERK1/2 signaling participates in up-regulating the expression of Rad51 and ERCC1 in NSCLC cells treated with capecitabine. Enforced expression of MKK1/2-CA could rescue the cytotoxicity induced by emodin and capecitabine. Consistent with our previous reports that the ERK1/2 activation is essential for Rad51 and ERCC1 induction, our results collectively indicate that inactivation of ERK1/2 signaling is critical in downregulating the expression of Rad51 and ERCC1 and in enhancing the capecitabine-induced cytotoxicity by emodin.

In this study, Rad51 play a protective role in cytotoxicity induced by capecitabine alone or combined with emodin.

Consistence with our studies, MP470 (a tyrosine kinase inhibitor of c-Met) was shown to inhibit Rad51 expression and enhance the radiosensitization of several glioblastoma multiforme (GBM) cell lines both *in vitro* and *in vivo* [38]. CP-751,871, a human monoclonal antibody specific to the insulin-like growth factor I receptor (IGF-1R), sensitizes NSCLC cells to radiation by inhibiting the Rad51-dependent repair of radiation-induced double-strand breaks [39]. Parsels et al. have shown that inhibition of checkpoint kinase 1 (Chk1) could sensitize pancreatic cancer cells to gemcitabine by inhibition of Rad51 protein expression [40]. Moreover, Rad51 siRNA transfection can enhance the chemosensitivity of cancer cells to cisplatin *in vitro* and *in vivo* [41]. In contrast, tumor cells with overexpressed Rad51 are more resistant to DNA damage induced by chemotherapy [42].

Metzger et al. has shown that ERCC1 mRNA levels had a statistically significant relationship to response and survival in patients with primary gastric adenocarcinoma treated with cisplatin and 5-FU [43]. Overexpression of ERCC1 has been reported to induce cisplatin-resistance in cancer cell lines [44,45], and high levels of ERCC1 mRNA are also associated with chemoresistance to oxaliplatin-based therapy in colorectal cancer [46]. SU5416, an inhibitor of vascular endothelial growth factor receptor, can enhance cisplatin-induced cytotoxicity through decreasing ERCC1 protein expression in human ovarian cancer cells [47]. Consistent with our study, knockdown of ERCC1 expression can increase the sensitivity of human lung cancer cells to capecitabine.

Emodin can enhance the sensitivity of cancer cells to chemotherapeutic agents [48]. For example, emodin and cisplatin combination remarkably elevate the reactive oxygen species level and enhance the chemo-sensitivity of DU-145 cells (a multidrug-resistant prostate carcinoma cell line) which compared to treatment with cisplatin-only [49]. In this study, we have shown that emodin can suppress the ERK1/2 activation, which is important for cell survival in NSCLC cells, and downregulate the induction of Rad51 and ERCC1 by capecitabine. In A549 lung carcinoma cells, emodin can suppress the cytoprotective signaling pathways of ERK1/2 and AKT, and can trigger mitochondrial dysfunction, cytochrome-c release from mitochondria, caspases activation and cell apoptosis [12]. Previous reports have also shown that emodin can result in an increase of PTEN phosphorylation followed by dephosphorylation of casein kinase 2 (CK2) and AKT in the SK-N-SH cells (neuroblastoma cells) [50]. Inhibition of CK2 by emodin could increase the apoptotic cell death in hepatocellular carcinoma and HeLa cells when treated with agonistic antibody of Fas or tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) [51]. Emodin also inhibits DNA synthesis and induces apoptosis in cervical cancer and ovarian cancer cells [52]. The generation of reactive oxygen species mediated by emodin inhibits the expression of pro-survival transcription factors NF- κ B (nuclear factor κ B), leading to cell death of HeLa cells [14,48]. Furthermore, emodin inhibits interleukin-6-induced JAK2 (Janus-activated kinase 2)/STAT3 (signal transducer and activator of transcription 3) pathway and induces apoptosis in myeloma cells via down-regulation of Mcl-1 (myeloid cell leukemia 1) [53]. Interestingly, our data showed that capecitabine-induced cytotoxicity can be enhanced in NSCLC through inhibiting the ERK1/2 signaling by emodin. Therefore, it is reasonable to consider emodin as a new potent anticancer agent for the treatment of NSCLC.

In advanced NSCLC, TP expression was associated with tumor response to capecitabine [34]. Therefore, in an attempt to increase the cytotoxic effect of capecitabine, several studies were conducted using capecitabine in combination with TP inducers. Previous clinical studies have shown that docetaxel can enhance the antitumor activity of capecitabine via enhancement of TP

expression [6,54]. The increased enzymatic activity of TP in cancer cells can activate capecitabine in cancer cell to treat metastatic breast and colorectal cancers [55], and the expression of TP is transcriptionally controlled by the Sp1 and STAT transcription factors [56,57]. Moreover, interferons induced TP expression via the JAK-STAT1 signaling pathway in malignant gliomas [57]. In this study, capecitabine combined with emodin exhibited a synergistic cytotoxic effect in NSCLC cells, which was correlated with the upregulation of TP expression. To our knowledge, our study is the first to demonstrate that emodin can enhance the capecitabine-induced cytotoxicity through increasing TP expression in an ERK1/2 independent manner.

In conclusion, enhancing the cytotoxicity to capecitabine by emodin is mediated by TP induction and down-regulation the expression of Rad51 and ERCC1. The combined treatment with emodin and capecitabine may be a novel and potential therapeutic modality for NSCLC in the future.

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