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ORIGINAL ARTICLE

Pemetrexed downregulates ERCC1 expression and enhances cytotoxicity effected by resveratrol in human nonsmall cell lung cancer cells

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Abstract The multitargeted antifolate pemetrexed has demonstrated certain clinical activities against nonsmall cell lung cancer (NSCLC). Resveratrol (3,5,4-trihydroxy-trans-stilbene) is a polyphenol found in grapes and other plants and has great potential as a preventative and therapeutic agent due to its anticarcinogenic activity. The efficacy of adding resveratrol to pemetrexed to prolong the survival of patients with NSCLC still remains unclear. The excision repair cross-complementation 1 (ERCC1) is a DNA repair gene coding 5′ endonuclease in nucleotide excision repair and is overexpressed in chemo- or radioresistant carcinomas. In this study, resveratrol (10–50 μM) inhibited cell survival in two NSCLC cells, H520 and H1975. Treatment with resveratrol increased ERCC1 messenger RNA and protein levels in a MKK3/6-p38 MAPK signal activation-dependent manner. Furthermore, blocking p38 MAPK activation by SB202190 or knocking down ERCC1 expression by transfection with small interfering RNA of ERCC1 enhanced the cytotoxicity of resveratrol. Combining resveratrol with pemetrexed resulted in a synergistic cytotoxic effect, accompanied with the reduction of phospho-p38 MAPK and ERCC1 protein levels, and a DNA repair capacity. Expression of constitutively active MKK6 (MKK6E) or HA-p38 MAPK vectors significantly rescued the decreased p38 MAPK activity, and restored ERCC1 protein levels and cell survival in resveratrol and pemetrexed cotreated NSCLC cells. In this study, for the first time, we have demonstrated the synergistic effect of combined treatment with resveratrol and pemetrexed in human NSCLC cells through downregulation of the MKK3/6-p38 MAPK-ERCC1 signal, suggesting a potential benefit of combining resveratrol and pemetrexed to treat lung cancer in the future.

Keywords ERCC1 · p38 MAPK · Resveratrol · Pemetrexed · Nonsmall cell lung cancer

Abbreviations

CFA	Colony-forming ability
ERCC1	Excision repair cross-complementation 1
ERK	Extracellular signal-regulated kinase
siRNA	Small interfering RNA
MAPK	Mitogen-activated protein kinase
NSCLC	Nonsmall cell lung cancer

Introduction

Lung cancer is a common malignant tumor worldwide, and nonsmall-cell lung cancer (NSCLC) is the most frequently seen type of lung cancer, accounting for approximately 85 % of all cases (Landis et al. 1999; Silvestri and Spiro 2006). The multitargeted antifolate pemetrexed is approved as a single agent in second-line treatment of patients with locally advanced or metastatic NSCLC after earlier chemotherapy (Hanna et al. 2004). Pemetrexed inhibits three of the enzymes essential for purine and pyrimidine synthesis, dihydrofolate reductase (DHFR), glycinamide ribonucleotide formyl transferase, and

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thymidylate synthase (TS) (Hanauske et al. 2001). Therefore, pemetrexed treatment depletes cellular ribonucleotide pools, modulates the cell cycle, and induces apoptosis (Tonkinson et al. 1999). However, resistance to pemetrexed may be extended, so combinations with other anticancer agents need to be investigated (Rodenbach et al. 2005).

The excision repair cross-complementing 1 (ERCC1) is a DNA damage repair gene that encodes 5′ endonuclease and is one of the key enzymes in nucleotide excision repair (Reardon et al. 1999; Niedernhofer et al. 2004). Clinical studies have found that a high level of ERCC1 has been associated with more efficient removal of DNA adducts induced by cisplatin, leading to resistance to platinum-based chemotherapy in human NSCLC (Metzger et al. 1998; Lord et al. 2002; Altaha et al. 2004). MAPKs pathways, including p38 MAPK, ERKs, and c-Jun N-terminal kinases are implicated in the response to chemotherapeutic drugs (Makin and Dive 2001). MAPKs pathway signaling modules consist of a three-tiered kinase core, where an MAPKKK activates a MAPKK (MKK), which in turn activates a MAPK (Lewis et al. 1998). The primordial MAPK cascades are ubiquitously expressed and respond to various external cues and drugs (Lewis et al. 1998). Previous studies have demonstrated that the anticancer agents cisplatin and etoposide induce ERCC1 expression in an ERK1/2 and p38 MAPK-dependent manner in NSCLC cells, respectively (Tsai et al. 2011, 2012).

Resveratrol (3,5,4′-trihydroxy-trans-stilbene), a polyphenol found in grapes and root extracts of *Polygonum cuspidatum*, has been demonstrated to exhibit antiproliferative, antioxidative, and anti-inflammatory properties (Kawada et al. 1998; ElAttar and Virji 1999; Wadsworth and Koop 1999). Resveratrol inhibits the growth of several human cancer cells, such as breast cancer cells (Joe et al. 2002; Pozo-Guisado et al. 2002), HL60 promyelocytic leukemia cells (Roberti et al. 2003), colorectal cells (Delmas et al. 2002), and epidermoid carcinoma cells (Ahmad et al. 2001). A recent study also suggested that resveratrol can chemosensitize breast cancer cells to chemotherapeutics drugs (Casanova et al. 2012). Resveratrol is associated with the regulation of cellular MAPKs pathways that are involved in cancer progression, apoptosis, and angiogenesis (She et al. 2001; Shih et al. 2002; Niles et al. 2003, Garvin et al. 2006).

In the current study, the detailed mechanism of resveratrol's role in regulating DNA repair protein ERCC1 expression and inducing the cytotoxic effect in human lung cancer cells was investigated.

Methods

Materials

Resveratrol, cycloheximide, actinomycin D, and tanshinone IIA (an AP-1-specific chemical inhibitor) were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Pemetrexed [L-glutamic acid, *N*-[4-[2-(2-amino-4,7-dihydro-4-oxo-1*H*-pyrrolo[2,3-D]pyrimidin-5-yl)ethyl]benzoyl]-, disodium salt, heptahydrate] was a gift from Eli Lilly Corporation (Indiana polis, IN, USA). SB202190 was purchased from Calbiochem-Novabiochem (San Diego, CA, USA).

Cell culture

Human lung carcinoma cells H520 and H1975 were obtained from the American Type Culture Collection (Manassas, VA, USA), and the cells were cultured at 37 °C in a humidified atmosphere containing 5 % CO_2 in RPMI-1640 complete medium supplemented with sodium bicarbonate (2.2 %, *w*/*v*), L-glutamine (0.03 %, *w*/*v*), penicillin (100 U/ml), streptomycin (100 μg/ml), and fetal calf serum (10 %). The cell lines were routinely tested to confirm that they were free of *Mycoplasma*.

Western blot analysis

Equal amounts of proteins from each set of experiments were subjected to Western blot analysis as previously described (Ko et al. 2008). The specific phospho-p38 MAPK (Thr180/Tyr182) and phospho-MKK3 (Ser189)/MKK6 (Ser207) antibodies were purchased from Cell Signaling (Beverly, MA, USA). Rabbit polyclonal antibodies against ERCC1(N-20) (sc-494), p38(C-20) (sc-535), HA(F-7) (sc-7392), MKK3(N-20) (sc-959), and Actin(I-19) (sc-1616) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Small interfering RNA knockdown

The sense-strand sequences of small interfering RNA (siRNA) duplexes were as follows: ERCC1, 5′- GGAGCUGGCUAAG AUGUGU -3′; p38, 5′- GAACUGCGGUUACUUAAAC −3′ (#1), 5′- AUGAAUGAUGGACUGAAAUGGUCUG −3′ (#2), and scrambled (as a control), 5′- UUCUCCGAACGUG UCACGUTT -3′ (Dharmacon Research, Lafayette, CO, USA). Cells were transfected with siRNA duplexes (200 nM) using Lipofectamine 2000 (Invitrogen) for 24 h.

Transient expression of MKK6E and HA-p38 MAPK vectors

Plasmids transfection of MKK6E (a constitutively active form of MKK6) and HA-p38 MAPK was achieved as previously described (Tsai et al. 2012). Human lung cancer cells growing exponentially (10^6) were plated for 18 h, and then the expression vectors were transfected into H520 or H1975 cells using Lipofectamine (Invitrogen). After expression for 24 h, the cells were treated with resveratrol and/or pemetrexed.

Quantitative real-time polymerase chain reaction (PCR)

Polymerase chain reactions (PCRs) were performed using an ABI Prism 7900HT according to the manufacturer's instructions. Amplification of specific PCR products was detected using the SYBR Green PCR Master Mix (Applied Biosystems). The designed primers in this study were ERCC1 forward primer, 5′- GGGTGACTGAATGTCTGACCA -3′, ERCC1 reverse primer, 5′- GGGTACTTTCAAGAAGGGCTC -3′; GAPDH forward primer, 5′- CATGAGAAGTATGACAACAGCCT -3′; GAPDH reverse primer, and 5′- AGTCCTTCCACGATACCA AAGT -3′. For each sample, the data were normalized to the housekeeping gene GAPDH.

Reverse transcription PCR (RT-PCR)

RNA was isolated from cultured cells using TRIzol (Invitrogen) according to the manufacturer's instructions. Reverse transcription PCR (RT-PCR) was performed with 2 μg of total RNA using random hexamers, in accordance with the Moloney murine leukemia virus reverse transcriptase complementary DNA (cDNA) synthesis system (Invitrogen). The final cDNA was used for subsequent PCRs. ERCC1 was amplified using primers with the sequence of 5′- CCCTGGGAATTTGGCGA CGTAA -3′ (forward) and 5′- CTCCAGGTACCGCCCAGCT TCC -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. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was amplified as an internal control. The GAPDH primers were 5′-CTACATGGTTTACATGTTCC-3′ (forward) and 5′-GTGAGCTTCCCGTTCAGCTCA-3′ (reverse). Expression of GAPDH was used as a control to measure the integrity of the RNA samples.

Cell viability assay

In vitro 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy phenol)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. Cells were cultured at 5,000 per well in 96-well tissue culture plates. To assess cell viability, drugs were added after plating. At the end of the culture period, 20 μl of MTS solution (CellTiter 96 Aqueous One Solution Cell Proliferation Assay; Promega, Madison, WI, USA) was added, the cells were incubated for a further 2 h, and the absorbance was measured at 490 nm using an enzyme-linked immunosorbent assay (ELISA) plate reader (Biorad Technologies, Hercules, CA, USA).

Combination index analysis

The cytotoxicity induced by the combined treatment with resveratrol and/or pemetrexed 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. The combination index analysis was performed using CalcuSyn software (Biosoft, Oxford, UK). The mean of CI values at a fraction affected of 0.50 were averaged for each experiment, and the values were used to calculate the mean between the three independent experiments.

Colony-forming ability assay

Immediately after drug treatment, the cells were washed with phosphate-buffered saline and trypsinized to determine the cell numbers. The cells were plated at a density of 500–1,000 cells on a 60-mm-diameter Petri dish in triplicate for each treatment and cultured for 12–14 days. The cell colonies were stained with 1 % crystal violet solution in 30 % ethanol. Cytotoxicity was determined by the number of colonies in the treated cells divided by the number of colonies in the untreated control.

Trypan blue dye exclusion assay

Cells were treated with resveratrol and/or pemetrexed for 24 h. Trypan blue dye can be excluded from living cells, but is able to penetrate dead cells. The proportion of dead cells was determined by hemocytometer, counting the number of cells stained with trypan blue.

AP-1 transcription activity ELISA

The nuclear fractions from the cells maintained in each culture condition were used to measure the AP-1 activity. AP-1 ELISA was performed using the AP-1 ELISA kit (Panomics Inc., Fremont, CA, USA) according to the manufacturer's protocol. Briefly, the 50 μg of nuclear fraction was mixed with transcription factor binding buffer supplied by the manufacturer and then applied to each well of a 96-well plate covered with an oligo-DNA fragment containing consensus AP-1 binding sequence. After incubation for 16 h at 4 °C without agitation, the wells were washed five times with 200 μl phosphate-buffered saline (PBS) containing 0.05 % Tween 20 (PBS-T). After the final wash, 100 μl of diluted anti-c-Jun antibody (1:100) solution was added to each well, except the blank wells, and the plate was incubated for 1 h at room temperature without agitation. Each well was washed again using PBS-T and then incubated with 100 μl of diluted peroxidase conjugated secondary antibody (1:100) for 1 h at room temperature without agitation. Each well was then treated with chemiluminescence developing solution. After 30-min incubation at room temperature with gentle agitation and protected from light, 100 μl of the stop solution was added to each well and absorbance was measured at a wavelength of 450 nm using a spectrophotometric plate reader (Benchmark Plus Microplate Spectrophotometer, Bio-Rad Laboratories, Inc.).

Host cell reactivation assay

For host cell reactivation assays, the pGL4.13-Luc plasmid was damaged in vitro by treatment with 250 nM cisplatin at 37 °C for 1 h in the dark. The pGL4.13-Luc plasmid (Promega) was used to estimate the capacity of cells to reactivate damaged plasmid. The undamaged plasmid was used to normalize for transfection efficiency. NSCLC cells were then transiently transfected with 1 μg of treated pGL4.13-Luc plasmid/well using the Lipofectamine (Invitrogen) Plus method following the manufacturer's instructions. In all cases, cells were collected 24 h after transfection, and cell extracts were used to determine luciferase activity. Luciferase activity values were quantified with a ZENYTH 3100 luminometer (Anthos Labtec Instruments Gmbh, Salzburg, Austria).

Luciferase assay

Cells were grown at 7×10^4 cells per well in six-well plates and were transfected with a pGL4.13-Luc vector. After treatment, cells were harvested by aspiration of the media followed by the addition of lysis buffer (Promega) per well and transferred to an opaque 96-well plate. Luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions and measured on a ZENYTH 3100 luminometer. Data are presented as fold increase over basal levels, based on the readings of the mean±standard error of the mean (SEM) of luminescence.

Statistical analyses

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

Results

Resveratrol inhibited cell survival of NSCLC cells

To investigate if resveratrol had any growth inhibitory activity against human NSCLC cells, cells were treated with 10, 20, 30, 40, and 50 μM of resveratrol for 24, 48, and 72 h. The cell proliferation rate and cell viability were assessed by MTS and trypan blue exclusion assays. In Fig. 1a and b, resveratrol induced a concentration- and time-dependent reduction in cell viability and proliferation in H520 and H1975 cells. At 30 μM, ~19.53 and ~34.75 % reductions in H1975 cell viability were observed at 24 and 48 h, respectively, as assessed by MTS assay (Fig. 1a). Colony-forming assays were conducted to investigate whether resveratrol affected long-term clonogenic cell survival. As shown in Fig. 1c, resveratrol treatment significantly suppressed the cell colony forming ability in H520 and H1975 cells.

Effects of resveratrol on ERCC1 protein and mRNA expression

Next, to determine whether ERCC1 expression was associated with the effects of resveratrol, we first assessed H520 or H1975 cells treated with resveratrol for 1–24 h or various concentractions of resveratrol for 24 h, and the RT-PCR and real-time PCR was used for determination of the ERCC1 messenger RNA (mRNA) level (Fig. 2a and b). In addition, protein levels of ERCC1 were determined by Western blot analysis (Fig. 2c, d). Resveratrol induced ERCC1 mRNA and protein expression in a time and dose-dependent manner; this was accompanied by an increase in phospho-MKK3/6-p38 MAPK protein levels (Fig. 2).

Next, we proposed that the activation of the MKK3/6-p38 MAPK pathway was involved in the upregulation of ERCC1 expression. To examine the role of p38 MAPK in the upregulation of ERCC1 by resveratrol, we employed the p38-specific inhibitor SB202190 or specific small interfering RNA (siRNA) duplexes. Once these cells were pretreated with p38 MAPK inhibitor, the increased ERCC1 protein and mRNA levels were abrogated by SB202190 in resveratrol-exposed H520 or H1975 cells (Fig. 3a, b). In addition, knockdown of the p38 MAPK expression by specific si-p38 RNA could attenuate the resveratrol-induced ERCC1 protein and mRNA levels (Fig. 3c, d). Therefore, we concluded that resveratrol increased ERCC1 expression in a p38 MAPK activation manner.

Previous study indicated that an increase in activating protein (AP)-1 transcriptional activity as a result of the expression of the oncogenic H-Ras was found to be crucial for ERCC1 induction in NIH3T3 and MCF-7 cells (Youn et al. 2004). Therefore, we tested whether resveratrol could increase the AP-1 activity using an AP-1 ELSA kit. Resveratrol treatment increased AP-1 activity in a dose-dependent manner in H520 and H1975 cells (Fig. 3e). Moreover, inhibition of AP-1 activity by tanshinone IIA (an AP-1-specific chemical inhibitor) could attenuate resveratrol-induced ERCC1 protein and mRNA expression (Fig. 3f–h).

Knockdown of ERCC1 NSCLC cells sensitized to resveratrol

We next examined the effect of siRNA-mediated ERCC1 knockdown on resveratrol-induced cytotoxicity and cell growth inhibition in NSCLC cells. At 24 h posttransfection, real-time RT-PCR and Western blot analysis showed that ERCC1 gene knockdown was accompanied by a decrease of ERCC1 mRNA

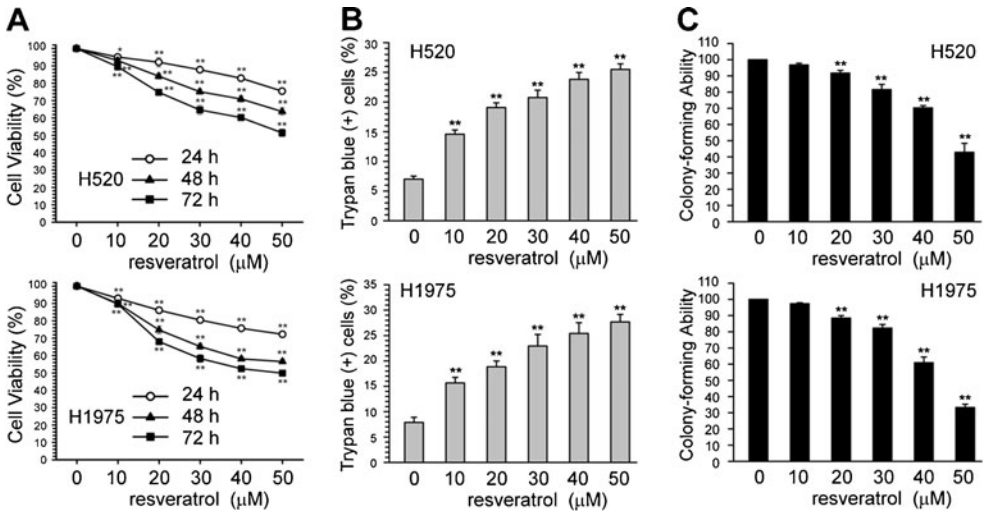

Fig. 1 Dose–response curves of resveratrol for cell survival in H520 or H1975 cells. **a** H520 or H1975 cells were treated with various concentrations of resveratrol (10–50 μM) for 24, 48, and 72 h. Cell survival was determined by MTS assay. **b** After cells were treated with various concentrations of resveratrol for 24 h, both unattached and attached cells were collected and stained with trypan blue dye, and the numbers of dead cells were manually counted. *Columns* percentage of trypan blue-positive cells, representing a population of dead cells; *bar* standard error (*SE*) from three independent experiments. **c** Resveratrol was added to cells for 24 h, cytotoxicity was determined by colony-forming ability assay. *$p<0.05$ and **$p<0.01$, respectively, using Student's *t* test for the comparison between the cells treated with or without resveratrol

and protein in untreated or resveratrol-treated H1975 and H520 cells; however, the resveratrol-induced p38 MAPK activation was not affected (Fig. 4a, b). Furthermore, suppression of ERCC1 protein expression by si-ERCC1 RNA resulted in increased sensitivity to resveratrol compared to si-control transfected cells (Fig. 4c). We also conducted a cell growth

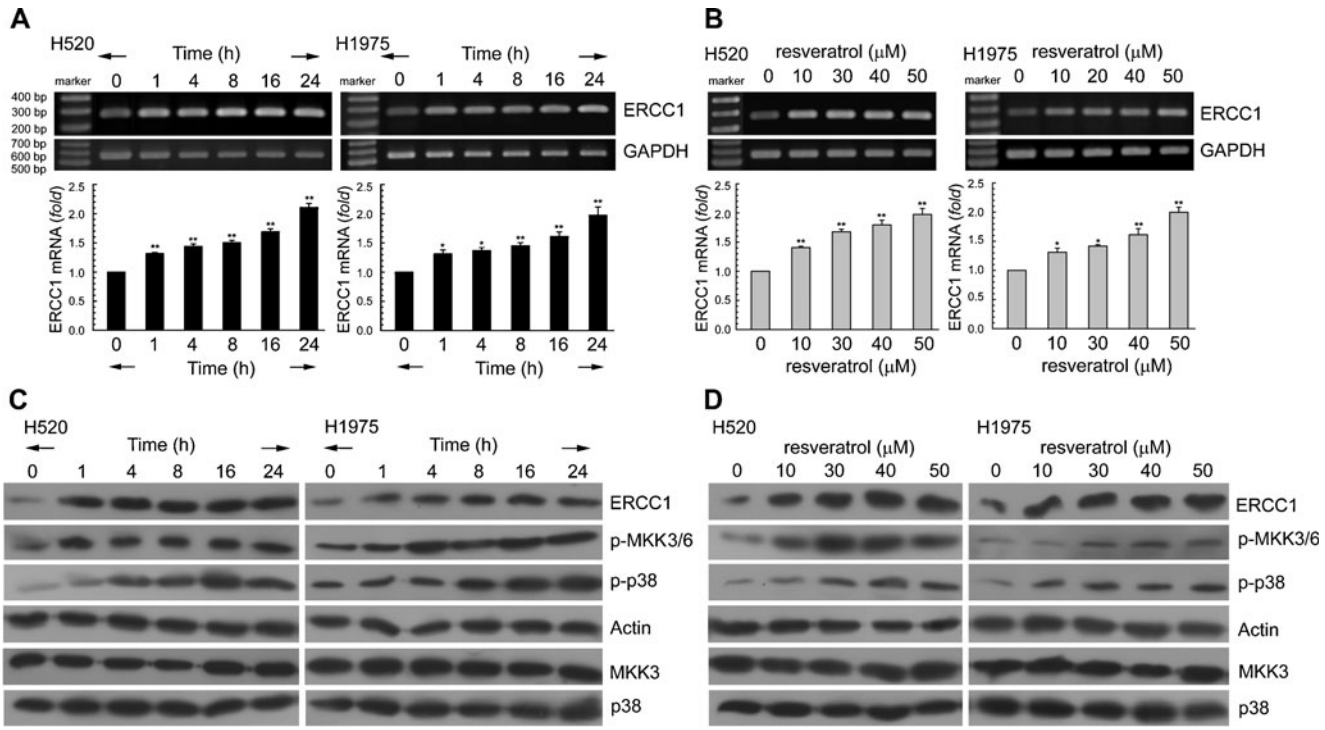

Fig. 2 Resveratrol increased ERCC1 expression in a dose and time-dependent manner. **a** H520 or H1975 cells (10^6) were cultured in complete medium for 18 h and then exposed to resveratrol (50 μM) for 1–24 h in complete medium. **b** Cells were treated with various concentrations of resveratrol for 24 h. After treatment, the total RNA was isolated and subjected to RT-PCR (*upper panel*) and real-time PCR (*lower panel*) for ERCC1 mRNA expression. The results (mean±SEM) were from three independent experiments. **$p<0.01$, *$p<0.05$ using Student's *t* test for the comparison between cells treated with or without resveratrol. **c**, **d** After treatment as above, the cell extracts were examined by Western blot for determination of ERCC1, phospho-MKK3/6, phospho-p38, MKK3, p38, and actin protein levels

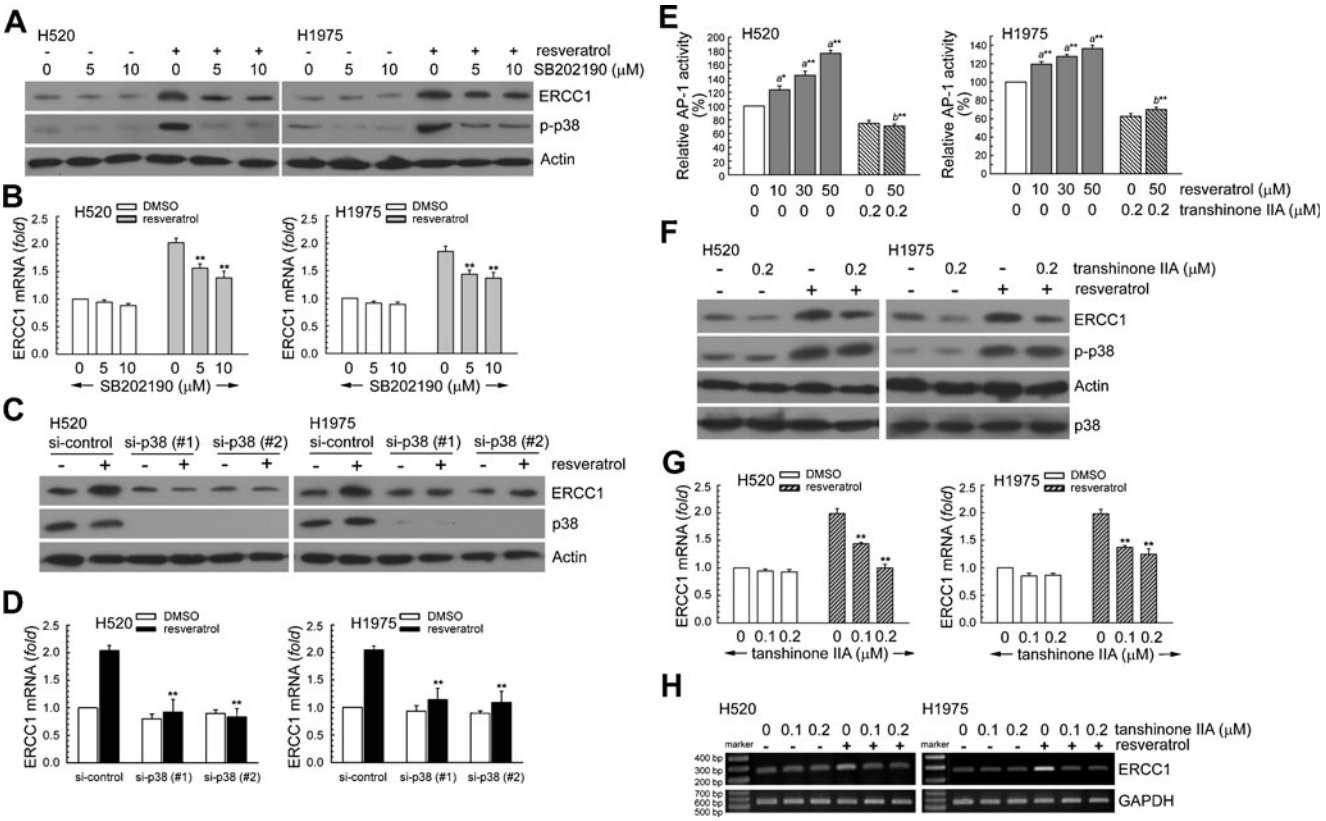

Fig. 3 Resveratrol increased ERCC1 expression via a p38 MAPK activation manner in NSCLC cells. **a**, **b** SB202190 (5 or 10 μM) was added to H520 or H1975 cells for 1 h before resveratrol (30 μM) treatment for 24 h. The results (mean±SEM) were from three independent experiments. $^{**}p<0.01$ using Student's *t* test for comparison between the cells treated with resveratrol–DMSO or resveratrol–SB202190 combination. **c**, **d** H520 or H1975 cells (5×10^5) were transfected with si-p38 RNA. After incubation for 24 h, the cells were treated with 30 μM resveratrol for 24 h. After treatment, the cell extracts were examined by Western blot (**a**, **c**) and real-time PCR (**b**, **d**) for determination of ERCC1 protein and mRNA levels, respectively. The results (mean±SEM) were from three independent experiments. $^{**}p<0.01$ using Student's *t* test for comparison between the cells treated with resveratrol in si-p38 RNA or si-scrambled RNA-transfected cells. **e** Tanshinone IIA (0.2 μM) was added to H520 or H1975 cells for 1 h before resveratrol treatment for 24 h. AP-1 transcription activity was determined by AP-1 transcription activity ELISA. $a^{**}p<0.01$ using Student's *t* test for comparison between the cells were treated with or without resveratrol. $b^{**}p<0.01$ using Student's *t* test for comparison between the cells treated with resveratrol alone or tanshinone IIA and resveratrol combination. **f**–**h** The cells pretreated with tanshinone IIA (0.1 and 0.2 μM) for 1 h were exposed to resveratrol (50 μM) for an additional 24 h. After treatment, the cell extracts were examined by Western blot (**f**) and real-time PCR (**g**) or RT-PCR (**h**) for determination of ERCC1 protein and mRNA levels, respectively. The results (mean±SEM) were from four independent experiments. $^{**}p<0.01$ using Student's *t* test for comparison between the cells treated with resveratrol alone or with the resveratrol–tanshinone IIA combination

inhibition assay to evaluate the synergistic effects of ERCC1 knockdown with resveratrol treatment. More inhibition of cell growth was induced by the combination of ERCC1 siRNA and resveratrol than by resveratrol alone in H520 or H1975 cells (Fig. 4d). Therefore, downregulation of ERCC1 expression could sensitize NSCLC cells to resveratrol.

Blocking p38 MAPK activation enhanced resveratrol-induced cytotoxicity

Next, to examine the role of p38 MAPK in the cytotoxic effect of resveratrol, we employed the si-p38 RNA to block resveratrol-induced p38 MAPK activation or p38 inhibitor SB202190. The p38 MAPK silencing by si-p38 RNA or cotreatment with SB202190 further decreased significantly cell viability in resveratrol-exposed H520 or H1975 cells, compared with resveratrol treatment alone (Fig. 5a, b). Moreover, inhibition of AP-1 activity by tanshinone IIA could further decrease cell viability under resveratrol treatment (Fig. 5c). Taken together, inactivation of the p38 MAPK-AP-1-ERCC1 signal could enhance resveratrol sensitivity in NSCLC cells.

Combination with pemetrexed enhanced the cytotoxic effect and growth inhibition of resveratrol

Preclinical studies suggested that the combinations of pemetrexed with cisplatin, as well as taxanes and gemcitabine, produce

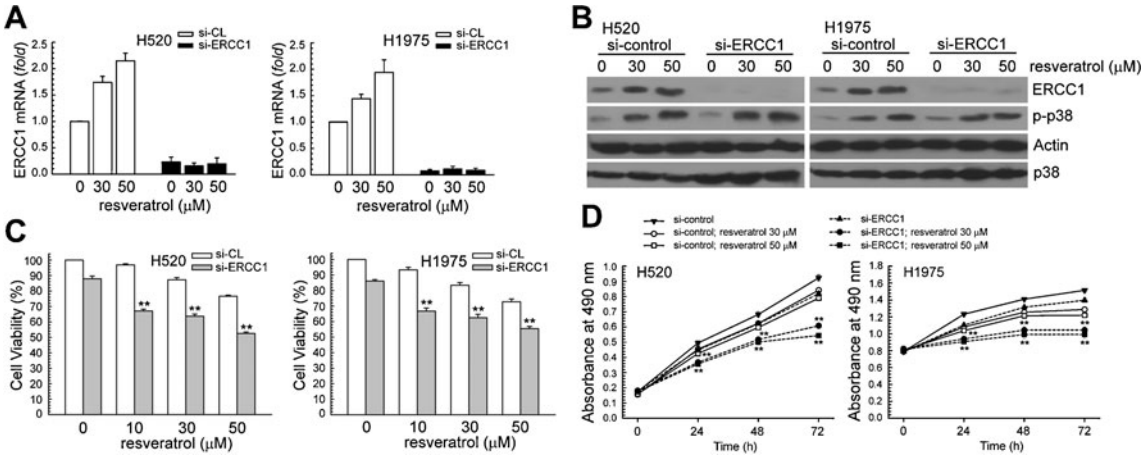

Fig. 4 Knockdown of ERCC1 expression by si-RNA transfection enhanced the cytotoxicity induced by resveratrol. **a**, **b** H520 or H1975 cells were transfected with siRNA duplexes (200 nM) specific to ERCC1 or scramble (control) in complete medium for 24 h prior to treatment with resveratrol (30 or 50 μM) in complete medium for 24 h; total RNA was isolated and subjected to real-time PCR for ERCC1 mRNA expression (**a**). Whole-cell extracts were collected for Western blot analysis using specific antibodies against ERCC1, phospho-p38, p38 MAPK, and actin (**b**). **c** After the above-mentioned treatment, cytotoxicity was determined by MTS assay. **d** After the cells were transfected with si-ERCC1 or si-scrambled RNA, the cells were either treated with resveratrol for 24, 48, and 72 h, after which living cells were determined by the MTS assay. The results (mean±SEM) were from three independent experiments. $^{**}p<0.01$ using Student's *t* test for comparison between the cells treated with resveratrol in si-ERCC1 RNA or si-scrambled RNA-transfected cells

additive or synergistic cytotoxicity (Teicher et al. 1999, 2000); therefore, we next attempted to determine whether pemetrexed sensitized NSCLC cells to the cytotoxic effects of resveratrol. The effect of the combination of pemetrexed and resveratrol on cell viability using MTS and trypan blue exclusion assays were examined. Pemetrexed and resveratrol were combined at a ratio of 1:5 or 1:2 and MTS assay was used to analyze cell viability. Use of the combination of pemetrexed plus resveratrol for 24 h resulted in a greater loss of cell viability in H520 and H1975 cells than that caused by either pemetrexed or resveratrol alone (Fig. 6a). The CI values for pemetrexed and resveratrol were <1, indicating synergism (Fig. 6b). In addition, H520 and H1975 cells were exposed to pemetrexed and/or resveratrol, and cell proliferation was determined within 1–4 days after exposure to the drugs. Pemetrexed and resveratrol cotreatment had a greater cell growth inhibition effect than either treatment alone (Fig. 6c). The results showed that pemetrexed enhanced the cytotoxic effects of resveratrol in human NSCLC cells.

Pemetrexed abrogated resveratrol-induced ERCC1 protein and upregulation of mRNA levels

In order to assess the mechanism of the synergistic effects, we hypothesized that pemetrexed would affect ERCC1 expression. To test this, H520 and H1975 cells were exposed to various concentrations of resveratrol (10, 30, and 50 μM) and pemetrexed (10 μM) for 24 h. Pemetrexed suppressed the endogenous and resveratrol-induced phospho-MKK3/6-p38 MAPK and ERCC1 protein levels (Fig. 7a). Moreover, the results from real-time PCR analysis showed that pemetrexed decreased resveratrol-induced ERCC1 mRNA level in H520 and H1975 cells (Fig. 7b).

Downregulation of ERCC1 expression by pemetrexed contributed to increased mRNA and protein instability in resveratrol-exposed NSCLC cells

Next, we examined the possible mechanisms for posttranscriptional regulation of ERCC1 transcripts under resveratrol and/or pemetexed treatment. To evaluate the stability of ERCC1 mRNA in resveratrol-exposed H520 or H1975 cells, we treated these cells with actinomycin D to block de novo RNA synthesis and then measured the levels of existing ERCC1 mRNA using real-time PCR (Fig. 7c; upper panel) and RT-PCR (Fig. 7c; lower panel) at 3, 6, and 9 h after treatment. After actinomycin D coexposure for 9 h, higher levels of ERCC1 mRNA remained with resveratrol treatment compared to untreated cells (Fig. 7c). Then, cycloheximide (an inhibitor of de novo protein synthesis) was added to resveratrol for 6, 12, and 18 h, and then the remaining ERCC1 protein was analyzed by Western blot. Resveratrol treatment significantly prevented ERCC1 degradation after cycloheximide treatment, compared to untreated cells (Fig. 7d). It was of interest that combination with pemetrexed suppressed resveratrol-induced ERCC1 mRNA and protein stability (Fig. 7c, d). These results indicated that pemetrexed alone inhibited ERCC1 protein production and repressed

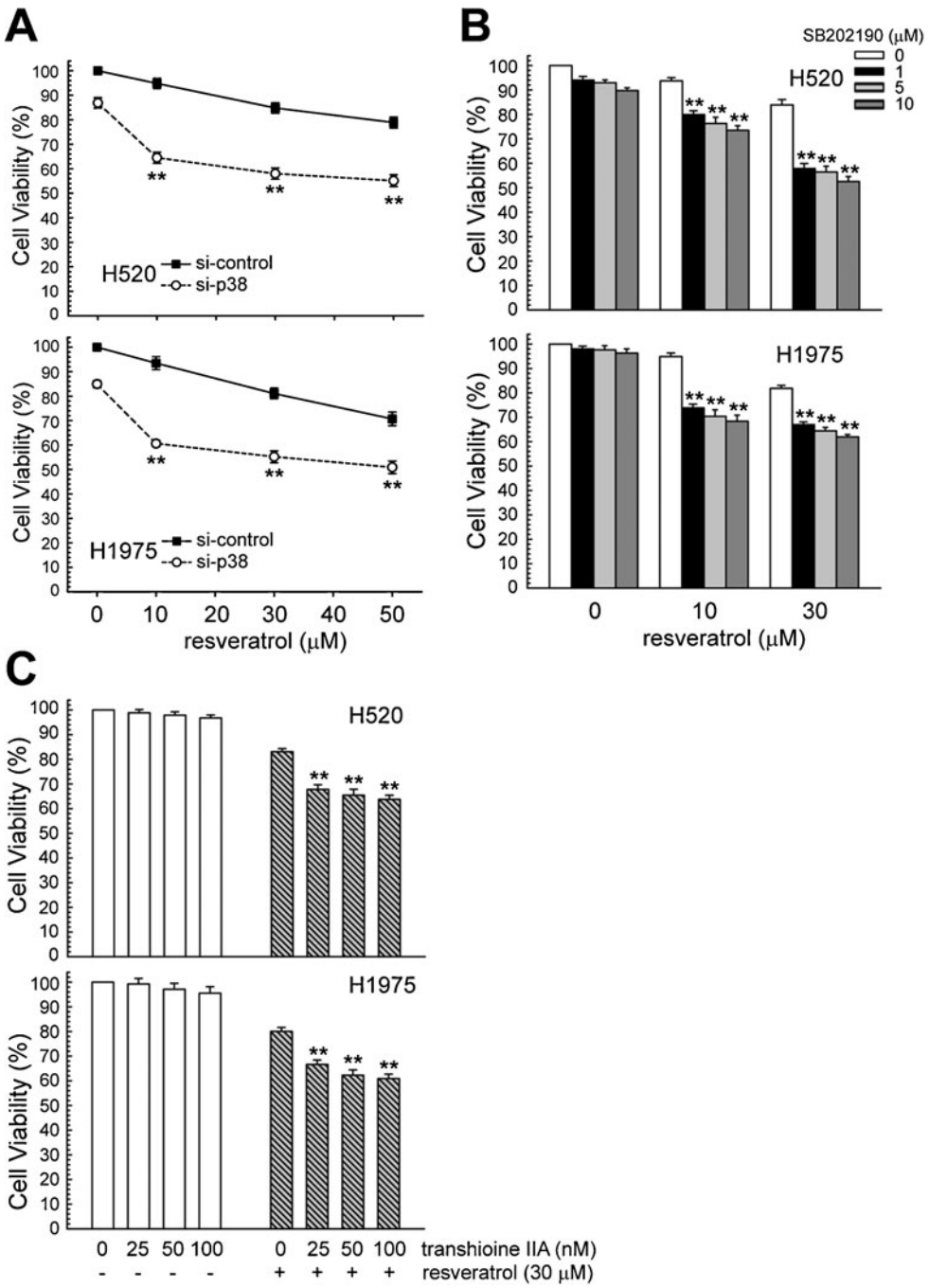

Fig. 5 Inhibition of p38 MAPK activation by specific si-p38 MAPK RNA transfection or SB202190 enhanced the cytotoxicity induced by resveratrol. **a** The si-p38 RNA-transfected cells were treated with resveratrol for 24 h. Cytotoxicity was determined by the MTS assay. $^{**}p<0.01$ using Student's *t* test for comparison between the cells treated with resveratrol in si-p38 RNA or si-scrambled RNA-transfected cells. **b** Cells were pretreated with SB202190 (1, 5, 10 μM) for 1 h and then co-treated with resveratrol (10, 30 μM) for 24 h. Cytotoxicity was determined by the MTS assay. $^{**}p<0.01$ using Student's *t* test for comparison between the cells treated with resveratrol alone or with the resveratrol–SB202190 combination. **c** Tanshinone IIA (25, 50, or 100 nM) was added to H520 or H1975 cells for 1 h before resveratrol (30 μM) treatment for 24 h. Cytotoxicity was determined as before. $^{**}p<0.01$, comparison between resveratrol alone and resveratrol/tanshinone IIA combination

resveratrol-induced ERCC1 expression by enhancing mRNA and protein instability.

Pemetrexed reduced cellular DNA repair capacity in resveratrol-exposed NSCLC cells

To investigate whether pemetrexed-mediated ERCC1 down-regulation has any effect on DNA repair capacity, we used the host cell reactivation of luciferase activity, which reflects the capacity of cells to repair plasmids damaged by cisplatin. The pGL4.13-Luc reporter plasmid was treated with 250 nM cisplatin. The NSCLC cells were transfected with cisplatin-treated pGL4.13-Luc plasmid and then coadded with resveratrol and/or pemetrexed. Resveratrol treatment was more capable of generating luciferase activity from the cisplatin-treated vector, compared to the control cells (Fig. 8a). In contrast, both

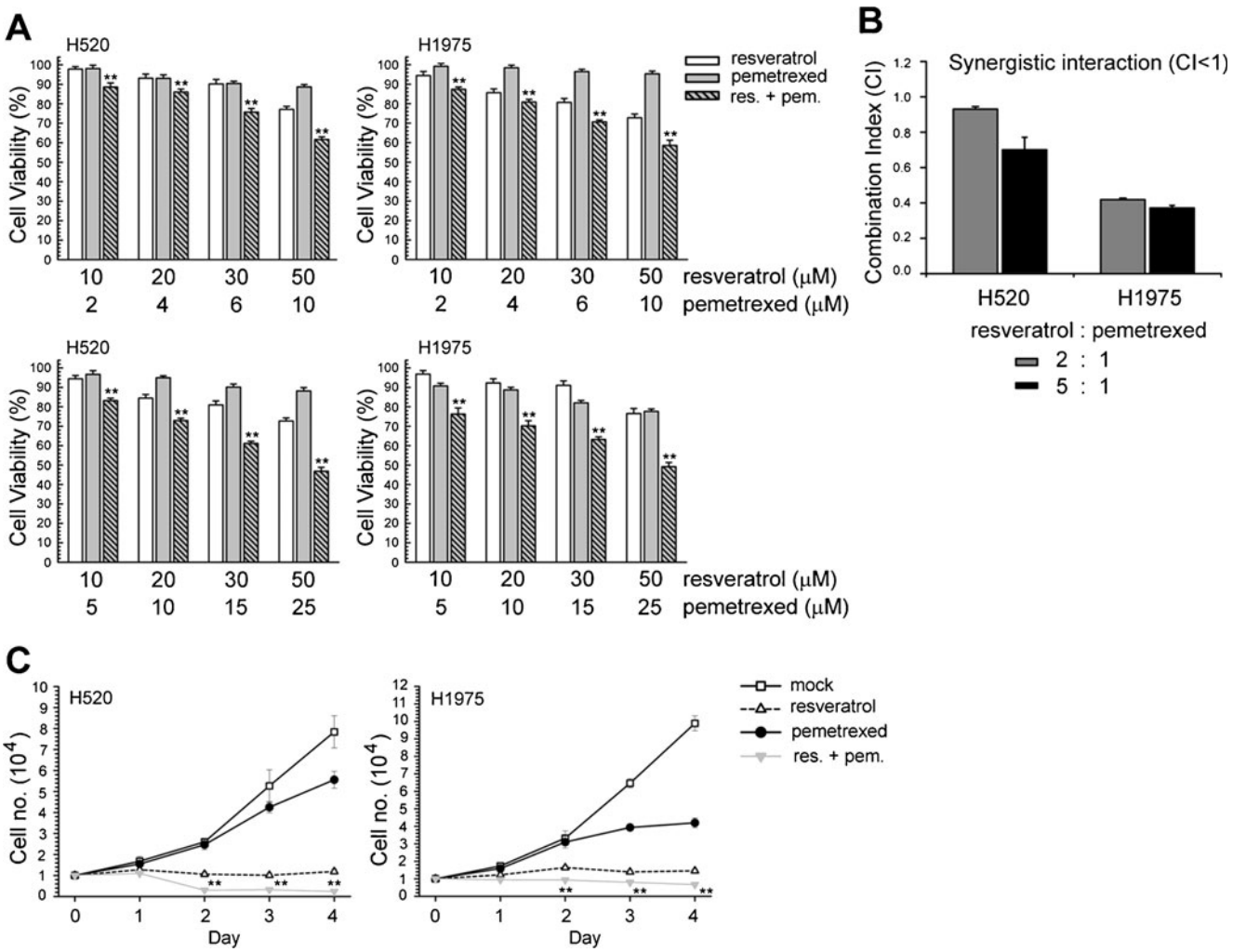

Fig. 6 Pemetrexed cotreatment with resveratrol synergistically enhances cytotoxicity. **a** Upper panel, resveratrol (10, 20, 30, and 50 μM) and/or pemetrexed (2, 4, 6, and 10 μM) were added to H520 or H1975 cells for 24 h. *Lower panel* Resveratrol (10, 20, 30, and 50 μM) and/or pemetrexed (5, 10, 15, and 25 μM) were added to H520 or H1975 cells for 24 h. Cytotoxicity was determined by assessment with the MTS assay. **b** The mean CI values at a fraction affected (*FA*) of 0.50 for resveratrol and metformin combined treatment were averaged for each experiment and used to calculate the mean between experiments. *Points and columns* Mean values obtained from three independent experiments; bars, standard error (*SE*). **c** Cells were treated with resveratrol (30 μM) and/or pemetrexed (6 μM) for 1–4 days after which living cells were determined by the trypan blue dye exclusion assay. $^{**}p<0.01$ using the Student's *t* test for comparison between cells treated with a drug alone or with a resveratrol/pemetrexed combination

cotreatment with pemtrexed and silencing of ERCC1 expression by specific siRNA could decrease DNA repair capacity in resveratrol-exposed H520 and H1975 cells (Fig. 8b, c).

Enhancement of p38 activation abrogated the synergistic cytotoxic effect and downregulated DNA repair capacity induced by combined pemetrexed and resveratrol

We then explored the roles of the p38 MAPK pathway directly affected by pemetrexed in the cellular response to resveratrol. H520 or H1975 cells were transfected with MKK6E or HA-p38 MAPK plasmids and then treated with pemetrexed and resveratrol. Transfection with either MKK6E or HA-p38 MAPK could enhance the ERCC1 protein level, DNA repair capacity, and cell survival that were suppressed by cotreatment with pemetrexed and resveratrol (Fig. 9). These results indicated that pemetrexed inhibited the p38 MAPK-mediated ERCC1 expression in NSCLC cells and consequently increased resveratrol-induced cytotoxicity.

Discussion

This study provides new insight into the mechanism underlying the role of p38 MAPK-mediated ERCC1 expression that protects the resveratrol-induced cyototoxicity in NSCLC cells. A previous study showed that in the highly invasive and metastatic breast cancer cell line MDA-MB-231, inhibition

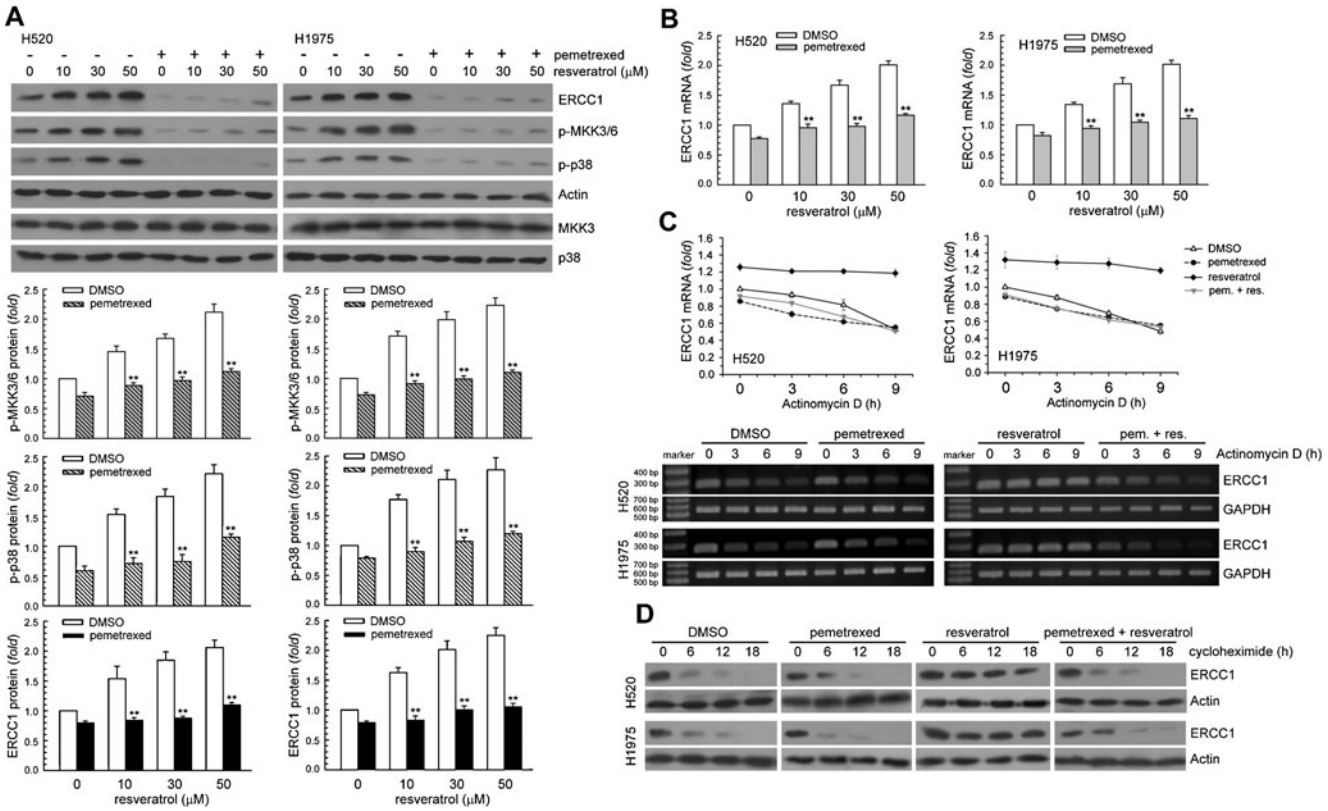

Fig. 7 Pemetrexed decreased resveratrol-induced ERCC1 protein and mRNA levels. **a** H520 or H1975 cells (10^6) were cultured in complete medium for 18 h and then were exposed to resveratrol (10, 30, 50 μM) and pemetrexed (10 μM) for 24 h. After treatment, cell extracts were examined by Western blot for the determination of ERCC1, phospho-p38, phospho-MKK3/6, and p38 MAPK, MKK3, and actin protein levels. **b** After treatment as above, total RNA was isolated and subjected to real-time PCR for ERCC1 mRNA expression. The means±standard deviation (*SD*) from four independent experiments. $^{**}p<0.01$, respectively, using Student's *t* test for the comparison between the cells treated with resveratrol/pemetrexed alone or combined treatment. Pemetrexed decreased ERCC1 mRNA and protein stability in resveratrol-treated NSCLC cells. **c** H520 or H1975 cells were exposed to resveratrol (30 μM) with pemetrexed (10 μM) or DMSO for 12 h in the presence or absence of actinomycin D (2 μg/mL) for 3, 6, or 9 h; total RNA was isolated and subjected to real-time PCR (*upper panel*) and RT-PCR (*lower panel*) for ERCC1 mRNA expression. ERCC1 mRNA levels were normalized against GAPDH in three separate treatments. **d** Cells were exposed to resveratrol (30 μM) and/or pemetrexed (10 μM) for 12 h followed by cotreatment with cycloheximide (CHX; 0.1 mg/mL) for 6, 12, or 18 h. Whole-cell extracts were collected for Western blot analysis

of ERK1/2 activation by its specific inhibitor or siRNA reverses the effect of resveratrol on Bcl-2 suppression and inhibits apoptosis, while overexpression of MEK1, which is directly upstream of both ERK1 and ERK2, enhances apoptosis induced by resveratrol (Nguyen et al. 2008). Herein, the results showed that phospho-p38 significantly increased in a dose- and time-dependent manner after resveratrol treatment in H520 and H1975 cells. The p38 MAPK inhibitor, SB202190, augmented the resveratrol-induced cytotoxicity in H520 and H1975 cells. Based on our results, we proposed a novel mechanism that enhances the resveratrol-induced cyototoxic effect on human lung cancer cells, which involves downregulation of MKK3/6-p38 signaling.

Pemetrexed is a multitargeted folate antimetabolite that primarily inhibits TS, DHFR, and glycinamide ribonucleotide formyltransferase (GARFT), with activity against a wide spectrum of tumor cell lines (Shih et al. 1997; Britten et al. 1999; Teicher et al. 2000). In the past decade, pemetrexed has had an increasingly established role in first-line, second-line, and maintenance therapy in the treatment of advanced NSCLC (Hanna et al. 2004; Scagliotti et al. 2008; Ciuleanu et al. 2009). Inhibition of TS and DHFR results in decreased thymidine, while inhibition of DHFR and GARFT results in disturbance of purine metabolism in DNA synthesis (Shih et al. 1997; Britten et al. 1999; Teicher et al. 2000). Preclinical studies demonstrated that the tumor growth inhibitory activities of pemetrexed combined with cisplatin, taxanes, and gemcitabine were generally additive or synergistic, and schedule dependent (Tonkinson et al. 1999; Teicher et al. 2000; Giovannetti et al. 2005). On the other hand, sustained activation of ERK1/2 and Cdk2/cyclin-A contributes to pemetrexed (0.1–1 μM)-induced S-phase arrest and apoptosis in human lung cancer A549 cells (Yang et al. 2011). Furthermore, in the presence of U0126 or PD98059 (MKK1/2 inhibitors) can block pemetrexed-triggered apoptosis in A549 cells (Yang et al. 2011). In contrast, pretreatment with a p38 MAPK

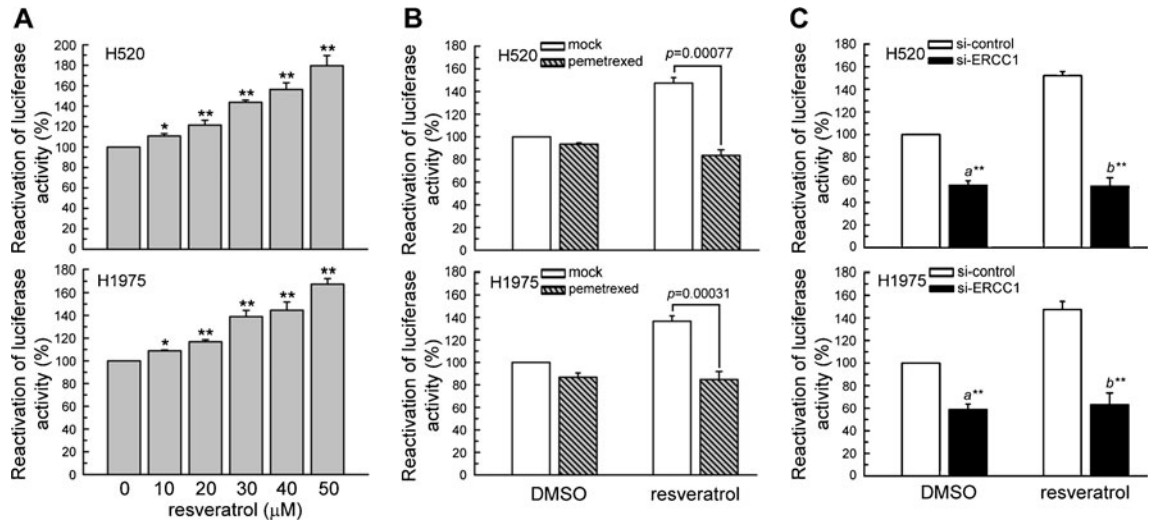

Fig. 8 Effects of ERCC1 on host cell reactivation capacity in resveratrol and /or pemetrexed-treated NSCLC cells. **a**, **b** Cells were transfected with 1 μg of cisplatin-treated pGL4.13-Luc reporter plasmid and then treated with various concentrations of resveratrol and/or pemetrexed (10 μM) for 24 h. The cell extracts were prepared for luciferase activity assays. **c** H520 or H1975 cells were transfected with si-ERCC1 RNA or si-control and then exposed to 30 μM resveratrol for 24 h. Then, the cells were transfected with 1 μg cisplatin-treated pGL4.13-Luc reporter plasmid. Cell extracts were prepared for luciferase activity assays. Each data bar is the mean of three independent experiments, and error bars represent SD. $a^{**}p<0.01$ using Student's *t* test for comparison between the cells transfected with si-ERCC1 RNA or si-scrambled RNA. $b^{**}p<0.01$ using Student's *t* test for comparison between the cells treated with resveratrol in si-ERCC1 RNA or si-scrambled RNA-transfected cells

inhibitor is not able to protect against pemetrexed-induced apoptotic cell death (Yang et al. 2011). This study indicated that cotreatment with pemetrexed (10 μM) could downregulate resveratrol-induced ERCC1 expression and p38 MAPK activation. Combined pemetrexed and resveratrol has synergistic cytotoxicity relative to NSCLC cells. However, whether

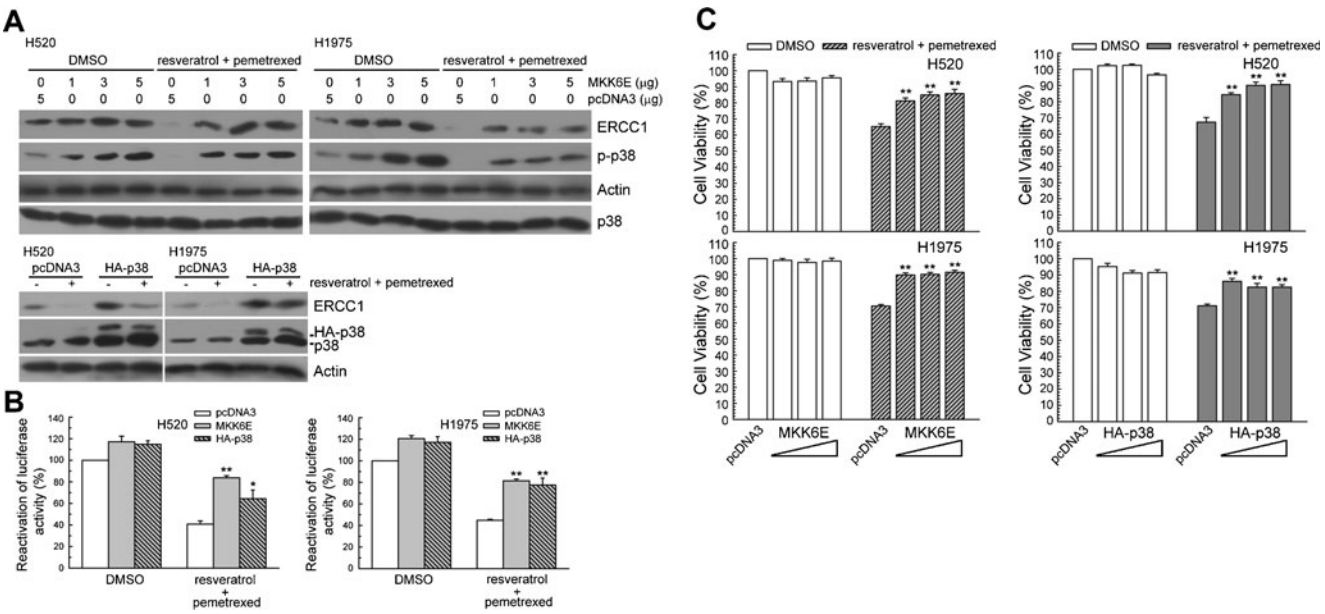

Fig. 9 Overexpression of MKK6E or HA-p38 MAPK restores pemetrexed-suppressed ERCC1 protein expression, DNA repair capacity, and cell survival in resveratrol-exposed H520 and H1975 cells. **a** MKK6E (1, 3, 5 μg) or HA-p38 MAPK (5 μg) or pcDNA3 (5 μg) expression plasmids were transfected into cells using lipofectamine. After expression for 24 h, the cells were treated with resveratrol (30 μM) and pemetrexed (10 μM) for an additional 24 h, and whole-cell extracts were collected for Western blot analysis. **b** H520 or H1975 cells were transfected with MKK6E (5 μg)/HA-p38 MAPK (5 μg)/ pcDNA3 (5 μg) and 1 μg cisplatin-treated pGL4.13-Luc reporter plasmid. Then, the cells were exposed to resveratrol (30 μM) and pemetrexed (10 μM) for 24 h. The cell extracts were prepared for luciferase activity assays. Each data bar is the mean of 3 independent experiments, and error bars represent SD. All *p* values were 0.05 versus the specific parental control. **c** After MKK6E or HA-p38 MAPK expression plasmids transfection, cells were treated with resveratrol (30 μM) and pemetrexed (10 μM) for 24 h. Cytotoxicity was determined by assessment with the MTS assay. $^{**}p<0.01$, $^{*}p<0.05$ by Student's *t* test for comparing cells treated with resveratrol and pemetrexed in MKK6E or HA-p38 MAPK vs. pcDNA3-transfected H520 or H1975 cells

ERK1/2 signal also involved in pemetrexed-induced synergistic cytotoxicity in resveratrol-exposed human lung cancer is worthy for further study.

In particular, pemetrexed acts on several pathways involved in DNA synthesis and cell death control, including the AKT pathway (Giovannetti et al. 2005). Pemetrexed caused a growth and survival response via epidermal growth factor receptor (EGFR)-mediated activation of the phosphatidylinositol 3-kinase/AKT (PI3K/AKT) pathway, which was inhibited by the EGFR tyrosine kinase inhibitor, erlotinib, and a specific phosphatidylinositol 3-kinase inhibitor, LY294002, in NSCLC cells. Therefore, a previous study suggested that inhibition of the PI3K/AKT pathway is a major determinant of synergism between pemetrexed and erlotinib (Li et al. 2007). However, whether the PI3K-AKT pathway is also involved in the synergistic cytotoxicity of resveratrol and pemetrexed is now under investigation by our team.

Taken together, our work is the first to identify ERCC1 induction by resveratrol through the MKK3/6-p38 MAPK pathway, and this phenomenon is required for NSCLC survival. Combination treatment with pemetrexed significantly decreased the expression of ERCC1 that is associated with enhancing the resveratrol-induced cytotoxic effect on NSCLC cells. These findings may have implications for the rational design of future drug regimens incorporating resveratrol and pemetrexed for the treatment of NSCLC.

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Conflict of interest The authors declare that they have no conflict of interest.

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