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Resveratrol Enhances Etoposide-Induced Cytotoxicity through Down-Regulating ERK1/2 and AKT-Mediated X-ray Repair Cross-Complement Group 1 (XRCC1) Protein Expression in Human Non-Small-Cell Lung Cancer Cells

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Abstract: Etoposide (VP-16), a topoisomerase II inhibitor, is an effective anti-cancer drug used for the treatment of non-small-cell lung cancer (NSCLC). Resveratrol is a naturally occurring polyphenolic compound that has been proved to have anti-cancer activity. XRCC1 is an important scaffold protein involved in base excision repair that is regulated by ERK1/2 and AKT signals and plays an important role in the development of lung cancer. However, the role of ERK1/2 and AKT-mediated XRCC1 expression in etoposide treatment alone or combined with resveratrol-induced cytotoxicity in NSCLC cells has not been identified. In this study, etoposide treatment increased XRCC1 mRNA and protein expression through AKT and ERK1/2 activation in two NSCLC cells, H1703 and H1975. Knockdown of XRCC1 in NSCLC cells by transfection of XRCC1 siRNA or inactivation of ERK1/2 and AKT resulted in enhancing cytotoxicity and cell growth inhibition induced by etoposide. Resveratrol inhibited the expression of XRCC1 and enhanced the etoposide-induced cell death and anti-proliferation effect in NSCLC cells. Furthermore, transfection with constitutive active MKK1 or AKT vectors could rescue the XRCC1 protein level and also the cell survival suppressed by co-treatment with etoposide and resveratrol. These findings suggested that down-regulation of XRCC1 expression by resveratrol can enhance the chemosensitivity of etoposide in NSCLC cells.

Lung cancer, the leading cause of cancer death in the world, is classified as non-small-cell lung cancer (NSCLC) and small-cell lung cancer [1]. NSCLC accounts for 85% of lung cancer cases, and despite aggressive radio- and/or chemotherapy, fewer than 20% of patients reach a 5-year survival. This poor treatment outcome is due to the primary or acquired drug resistance of NSCLC cells to present cytotoxic therapeutic agents [2,3].

Etoposide is an epipodophyllotoxin employed in the therapy of a wide spectrum of cancers [4–6]. *In vitro* studies have shown that etoposide increases topoisomerase II-mediated DNA breakage primarily by inhibiting the ability of the enzyme to religate cleaved nucleic acid molecules [7]. XRCC1 (X-ray repair cross-complementing group 1) is a key mediator of base excision repair (BER). Deficiency of XRCC1 in mice results in embryonic lethality [8,9]. XRCC1 interacts with enzymatic factors such as polyadenosine diphosphate (ADP)-ribose polymerase, DNA ligase III and DNA polymerase β to facilitate efficient repair of DNA single-strand breaks (SSBs) [10]. Down-regulation of XRCC1 expression in human breast cancer cell lines resulted in decreased SSB repair capacity and hypersensitivity to methyl methane sulphonate (MMS) [11]. A previous study has shown that the PI3K-AKT pathway regulates the basal expression of XRCC1 in non-irradiated cells, and MKK1/2-ERK1/2 is essential for the induction of XRCC1 after exposure to radiation [12]. However, whether ERK1/2 and AKT signals involve in regulating XRCC1 expression upon etoposide treatment and its role in the etoposide-induced cytotoxicity in NSCLC cells is still unclear.

Resveratrol (3,5,4′-trihydroxy-trans-stilbene) is a small-molecule natural component of grapes and red wine that has been shown to be a potential anti-cancer and chemopreventive agent [13–15]. A previous study suggested that resveratrol can chemosensitize breast cancer cells to chemotherapeutics drugs [16]. Recently, Sprouse *et al.* [17] study demonstrated that resveratrol treatment reduced cell proliferation and colony formation and increased senescence and apoptosis in paclitaxel-resistant triple-negative breast cancer cells. Combined treatment of resveratrol to benzo[*a*]pyrene-treated animals resulted in a decrease in p53 hyperphosphorylation and cell proliferation in the benzo[*a*]pyrene-treated mice [18]. However, whether resveratrol could enhance the etoposide-induced cytotoxic effect in NSCLC has never been studied.

In this study, we wanted to explore the molecular mechanism of etoposide in regulating XRCC1 expression in human lung cancer cells. We also wanted to know whether resveratrol

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could affect the sensitivity of cells to etoposide through modulating expression of XRCC1. These results may provide a rational design for future drug regimens incorporating etoposide and resveratrol for the treatment of NSCLC by modulating XRCC1 expression.

Materials and Methods

Chemicals. Etoposide, resveratrol, actinomycin D and cycloheximide were purchased from Sigma Chemical (St. Louis, MO, USA). LY294002 and U0126 were purchased from Calbiochem-Novabiochem (San Diego, CA, USA).

Cell culture. Human lung carcinoma cells H1703 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 units/ml), streptomycin (100 μg/ml) and foetal calf serum (10%). The cell lines were routinely tested to confirm that they were free of *Mycoplasma*.

Western blot analysis. After treatment, cells were rinsed twice with cold PBS and lysed in a whole-cell extract buffer (20 mM HEPES at pH 7.6, 75 mM NaCl, 2.5 mM MgCl2, 0.1 mM EDTA, 0.1% Triton X-100, 0.1 mM Na_3VO_4, 50 mM NaF, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin and 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride). The cell lysate was rotated at 4°C for 30 min. and then centrifuged at 9,279 g for 15 min., after which the precipitates were discarded. The BCA protein assay kit (Pierce, Grand Island, New York, USA) was employed to determine protein concentrations using bovine serum albumin as a standard. Equal amounts of proteins (50 μg) from each set of experiments were subjected to Western blot analysis. The membrane blocking and diluting antibodies were in skim milk. Antibodies were stripped from polyvinylidene difluoride membranes using a solution containing 2% SDS, 62.5 mM Tris-HCl, pH 6.8 and 0.7% (w/w) β-mercaptoethanol at 50°C for 15 min. before re-probing with another primary antibody. The specific phospho-AKT (Ser^{473}) (#9271) and phospho-ERK1/2 (Thr^{202}/Tyr^{204}) (#9101) antibodies were purchased from Cell Signaling (Beverly, MA, USA). Rabbit polyclonal antibodies against XRCC1 (H-300) (sc-11429), AKT (H-136) (sc-8312), 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).

Transfection with AKT-CA, MKK1-CA vectors, small interfering RNA. Exponentially growing human lung cancer cells (10^6) were plated for 18 hr and then constitutively active AKT expression plasmid (AKT-CA), which harboured a consensus myristylation domain that replaced the 4–129 amino acids of wild-type AKT, and MKK1-CA (a constitutively active form of MKK1) expression vectors were transfected into H1703 or H1975 cells using Lipofectamine (Invitrogen, Grand Island, New York, USA). The XRCC1 siRNA (sc-36859) or scramble control siRNA were obtained from Santa Cruz Biotechnology. Cells were transfected with siRNA duplexes (200 nM) for 24–48 hr using Lipofectamine 2000 (Invitrogen) for 24 hr.

Quantitative real-time polymerase chain reaction. Polymerase chain reaction (PCR) were performed using an ABI Prism 7900HT according to the manufacturer's instructions. Amplification of specific PCR products was performed using the SYBR Green PCR Master Mix (Applied Biosystems, Grand Island, New York, USA). For each sample, the data were normalized to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (*gapdh*). The designed primers in this study were as follows: *XRCC1* forward primer, 5′- GGGACCGGGTCAAA ATTGTT -3′; *XRCC1* reverse primer, 5′- ACCGTACAAAACTCAAG CCAAAG -3′; *gapdh* forward primer, 5′- CATGAGAAGTATGAC AACAGCCT -3′; *gapdh* reverse primer, 5′- AGTCCTTCCACGATAC CAAAGT -3′. Analysis was performed using the comparative Ct value method. For each sample, the data were normalized to the housekeeping gene *gapdh*.

MTS assay. The MTS tetrazolium compound (Owen's reagent) is bioreduced by cells into a coloured formazan product that is soluble in culture medium. This conversion is presumably accomplished by NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells. Cells were cultured at 5000 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 hr, and the absorbance was measured at 490 nm using an ELISA plate reader (Bio-Rad Technologies, Hercules, CA, USA).

Combination index analysis. The cytotoxicity induced by the combined treatment with etoposide and/or resveratrol 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 (FA) of 0.90, 0.75 and 0.50 was 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 500–1000 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 etoposide and/or resveratrol for 24 hr. Trypan blue dye can be excluded from living cells but is able to penetrate dead cells. The proportion of dead cells was determined by haemocytometer, counting the number of cells stained with trypan blue.

Statistical analyses. For each protocol, three or four independent experiments were performed. Results were expressed as the mean ± S.E.M. Statistical calculations were performed using SigmaPlot 2000 software (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

Etoposide increased phospho-ERK1/2, phospho-AKT, XRCC1 protein and mRNA expression in H1703 and H1975 cells.

To determine whether etoposide treatment could affect XRCC1 expression, we assessed H1703 or H1975 cells treated with etoposide (50 μM) for 1–24 hr or etoposide (10, 20, 30, 40, 50 μM) for 24 hr, and real-time PCR was used for the

determination of the XRCC1 mRNA level. The protein levels of XRCC1 were determined by Western blot analysis. In fig. 1A,B, etoposide induced XRCC1 mRNA and protein expression in a time- and dose-dependent manner and also increased phospho-ERK1/2 and phospho-AKT protein levels. In addition, to determine whether the activation of ERK1/2 and AKT was involved in up-regulation of XRCC1 by etoposide, H1703 or H1975 cells were pre-treated with MKK1/2 inhibitors (U0126) or PI3K inhibitors (LY294002) to block etoposide-induced ERK1/2 and AKT activation. In fig. 1C,D, as expected, the addition of U0126 or LY294002 decreased cellular and etoposide-induced ERK1/2 and AKT activation, respectively. Moreover, both U0126 and LY294002 pre-treatment decreased XRCC1 protein and mRNA levels in etoposide-exposed cells. Therefore, etoposide-increased XRCC1 protein and mRNA expression were correlated with ERK1/2 and AKT activation.

Inactivation of ERK1/2 and AKT activity enhanced XRCC1 mRNA and protein instability upon etoposide treatment.

Next, we examined whether ERK1/2 or AKT signal was involved in post-transcriptional regulation of XRCC1 transcripts under etoposide treatment. To evaluate the stability of XRCC1 mRNA in etoposide-exposed H1703 or H1975 cells, we treated the cells with actinomycin D to block *de novo* RNA synthesis, and then measured the levels of existing XRCC1 mRNA using real-time PCR at 4, 8 and 12 hr after treatment. In fig. 2A, after actinomycin D co-treatment, U0126 or LY294002 pre-treatment could enhance XRCC1 mRNA instability in etoposide-exposed H1703 and H1975 cells. Then, cycloheximide (an inhibitor of *de novo* protein synthesis) was added to etoposide treatment for 4, 8 and 12 hr, and the remaining XRCC1 protein was analysed by Western blot. In fig. 2B, less XRCC1 protein remained with U0126 or LY294002 co-treatment, compared with etoposide

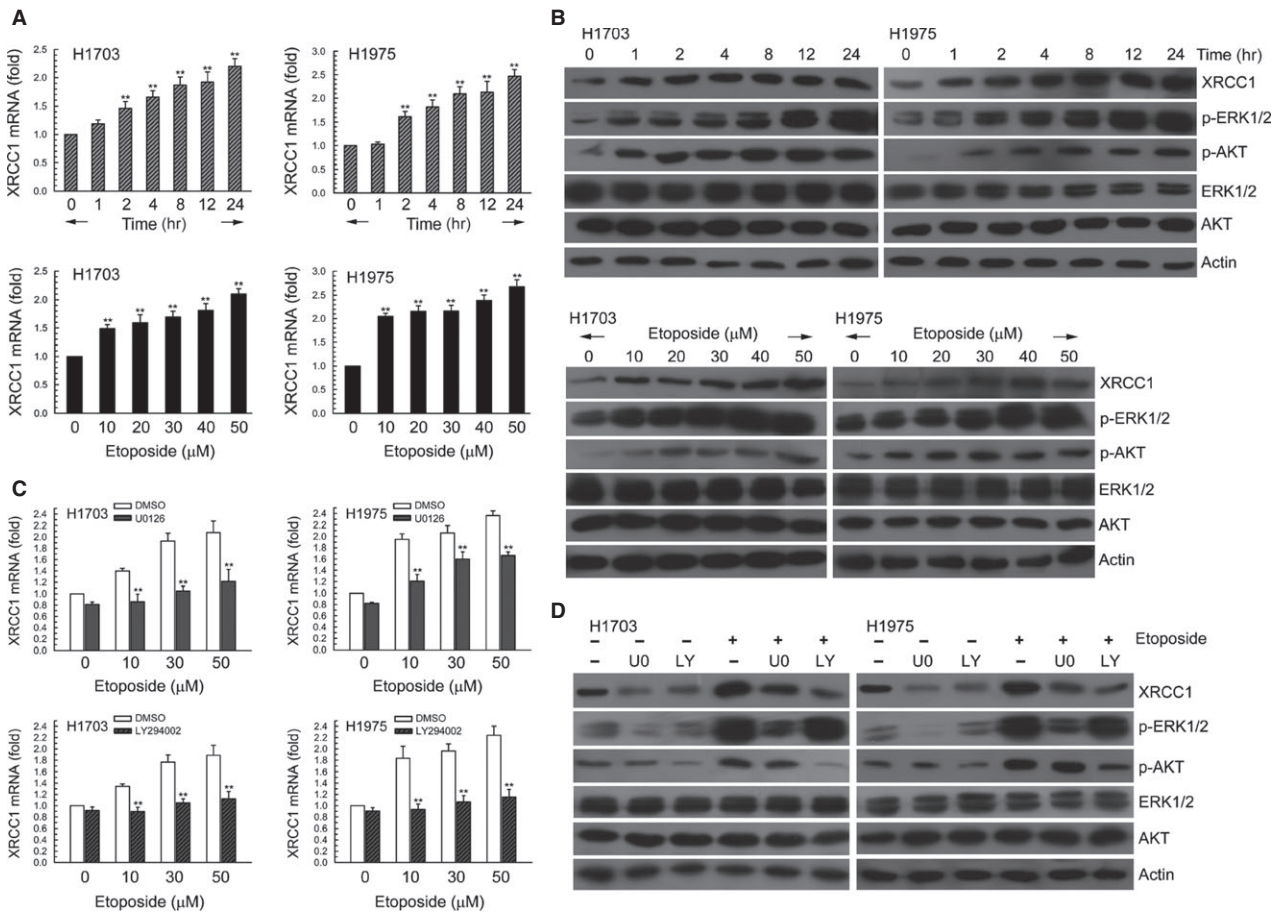


Fig. 1. Etoposide increased XRCC1 expression in a dose- and time-dependent manner. (A) H1703 or H1975 cells (10^6) were cultured in complete medium for 18 hr and then exposed to etoposide (50 μM) for 1, 2, 4, 8, 12 or 24 hr or various concentrations of etoposide (10–50 μM) for 24 hr in complete medium. The total RNA was isolated and subjected to real-time polymerase chain reaction (PCR) for XRCC1 mRNA expression. The results (mean ± S.E.M.) were from three independent experiments. (B) After treatment, the cell extracts were examined by Western blot for the determination of XRCC1, phospho-ERK1/2, phospho-AKT, ERK1/2, AKT and actin protein levels. (C) U0126 (5 μM) or LY294002 (5 μM) was added to H1703 or H1975 cells for 1 hr before etoposide treatment for 24 hr. The total RNA was isolated and subjected to real-time PCR for XRCC1 mRNA expression. The results (mean ± S.E.M.) were from three independent experiments. $^{**}p < 0.01$ using Student's *t*-test for comparison between the cells treated with etoposide–DMSO or an etoposide–U0126/LY294002 combination. (D) After treatment as above, the cell extracts were examined by Western blot.

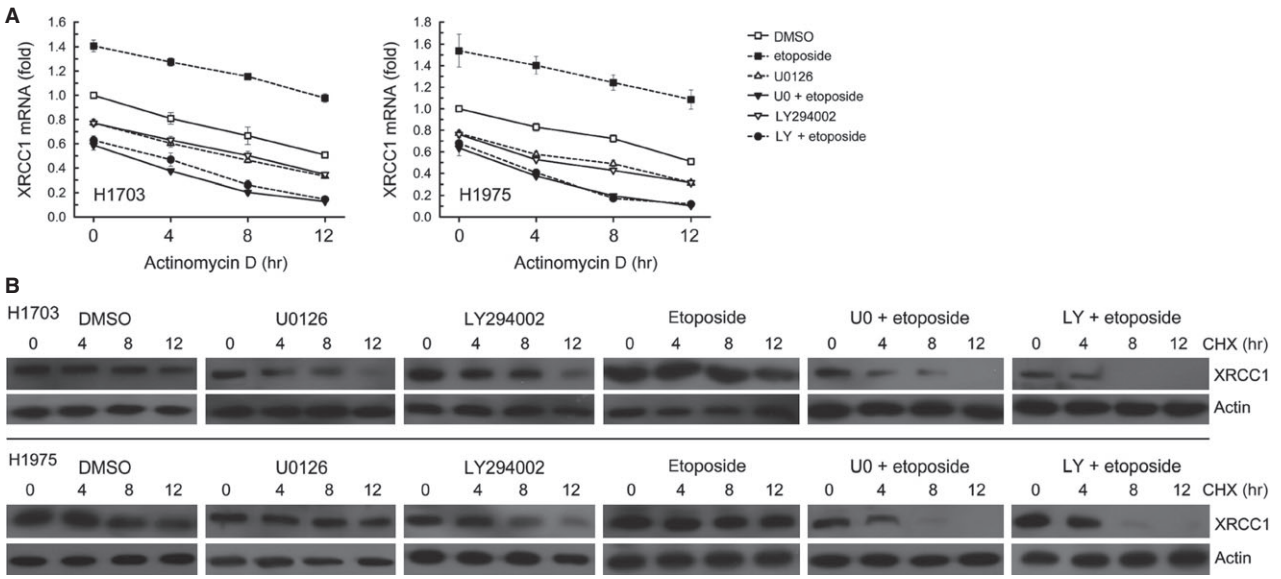


Fig. 2. Etoposide decreased XRCC1 mRNA and protein stability in non-small-cell lung cancer (NSCLC) cells. (A) H1703 or H1975 cells were exposed to etoposide (10 μM) and/or U0126/LY294002 for 12 hr in the presence or absence of actinomycin D (2 μg/ml) for 4, 8 or 12 hr; total RNA was isolated and subjected to real-time polymerase chain reaction (PCR) for XRCC1 mRNA expression. (B) Cells were exposed to etoposide (10 μM) for 12 hr followed by co-treatment with cycloheximide (CHX; 0.1 mg/ml) for 4, 8 or 12 hr. Whole-cell extracts were collected for Western blot analysis.

alone. Therefore, we concluded that inactivation of ERK1/2 and AKT activity decreased etoposide-induced XRCC1 expression via promoting mRNA and protein instability in H1703 and H1975 cells.

Knockdown of XRCC1 or blocking AKT and ERK1/2 activation enhanced etoposide-induced cytotoxicity and growth inhibition in NSCLC cells.

We next examined the effect of siRNA-mediated XRCC1 knockdown on etoposide-induced cytotoxicity and cell growth inhibition in NSCLC cells. At 24 hr post-transfection, real-time PCR and Western blot analysis showed a decrease in XRCC1 mRNA and protein in etoposide-treated H1703 and H1975 cells (fig. 3A). Furthermore, suppression of XRCC1 protein expression by si-XRCC1 RNA resulted in increased sensitivity to etoposide compared with si-control-transfected cells (fig. 3B,C). We also conducted a cell growth inhibition assay to evaluate the synergistic effects of XRCC1 knockdown with etoposide treatment. More inhibition of cell growth was induced by the combination of XRCC1 siRNA and etoposide than by etoposide alone in H1703 or H1975 cells (fig. 3D). Therefore, down-regulation of XRCC1 expression could enhance etoposide-induced cytotoxicity and growth inhibition in NSCLC cells.

Next, the role of ERK1/2 and AKT in the cytotoxic effect of etoposide was examined; the MKK1/2 inhibitor U0126 and PI3K inhibitor LY294002 were added to block ERK1/2 and AKT activation, respectively. Co-treatment with U0126 or LY294002 further decreased cell viability significantly and enhanced the anti-proliferative effect in etoposide-exposed H1703 or H1975 cells, compared with etoposide treatment alone (fig. 3E–G). Taken together, inactivation of the MKK1/2-ERK1/2 and PI3K-AKT signals could enhance etoposide-induced cytotoxicity in NSCLC cells.

Combination treatment with resveratrol enhanced the cytotoxic effect and growth inhibition of etoposide.

It has previously been shown that resveratrol can sensitize HepG2 and HCT-116 (liver and colon cancer cells) to etoposide and augments the anti-proliferative effect of chemotherapeutic drug mitomycin C (MMC) on human colorectal cancer cells [19,20]; therefore, we attempted to determine whether resveratrol could enhance the cytotoxic effects of etoposide in NSCLC cells. The effect of combined treatment with resveratrol and etoposide on cell viability was examined by MTS and trypan blue exclusion assays. Combined treatment with resveratrol and etoposide for 24 hr resulted in a greater loss of cell viability in H1703 and H1975 cells than treatment with either resveratrol or etoposide alone (fig. 4A,B). Etoposide and resveratrol were combined at a ratio of 1:5 or 1:2, and MTS assay was used to analyse cell viability (fig. 4C). The CI values for etoposide and resveratrol were <1, indicating the combination treatment had a synergistic effect (fig. 4C). In addition, H1703 and H1975 cells were exposed to etoposide and/or resveratrol, and cell proliferation was determined 1–4 days after exposure to the drugs. Etoposide and resveratrol co-treatment had a greater cell growth inhibition effect than either treatment alone (fig. 4D). Colony-forming assays were conducted to investigate whether resveratrol affected etoposide-mediated long-term clonogenic cell survival in NSCLC cells. As shown in fig. 4E, resveratrol significantly suppressed the cell colony-forming ability of etoposide-exposed H1703

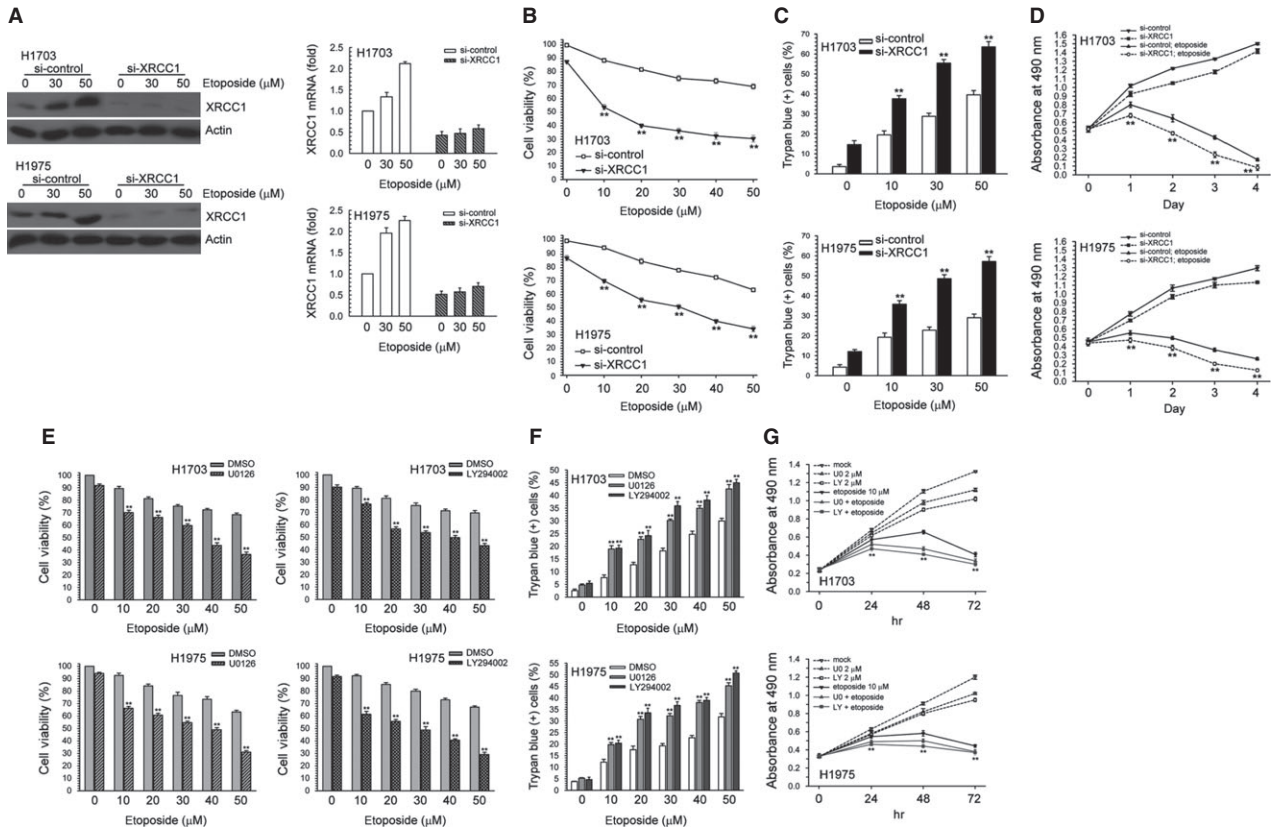


Fig. 3. Knockdown of XRCC1 expression by siRNA transfection enhanced the cytotoxicity induced by etoposide. (A) H1703 or H1975 cells were transfected with siRNA duplexes (200 nM) specific to XRCC1 or scrambled (control) in complete medium for 24 hr prior to treatment with etoposide in complete medium for 24 hr; total RNA was isolated and subjected to real-time polymerase chain reaction (PCR) for XRCC1 mRNA expression (left panel). Whole-cell extracts were collected for Western blot analysis using specific antibodies against XRCC1 (right panel). (B and C) After the above-mentioned treatment, cytotoxicity was determined by the MTS assay (B) and trypan blue dye exclusion assay (C). (D) After the cells were transfected with si-XRCC1 or si-scrambled RNA, the cells were treated with etoposide (10 μM) for 1–4 days, after which living cells were determined by MTS assay. The results (mean ± S.E.M.) were from three independent experiments. $^{**}p < 0.01$ using Student's *t*-test for comparison between the cells treated with etoposide in si-XRCC1 RNA or si-scrambled RNA-transfected cells. (E and F) H1703 or H1975 cells were pre-treated with U0126 (5 μM) or LY294002 (5 μM) for 1 hr and then co-treated with etoposide for 24 hr. Cytotoxicity was determined by MTS assay (E) and trypan blue dye exclusion assay (F). (G) Cells were pre-treated with U0126 (2 μM) or LY294002 (2 μM) and/or etoposide (10 μM) for 24, 48 and 72 hr, after which living cells were determined by MTS assay. $^{**}p < 0.01$ using Student's *t*-test for comparison between the cells pre-treated with or without U0126/LY294002 in etoposide-exposed cells.

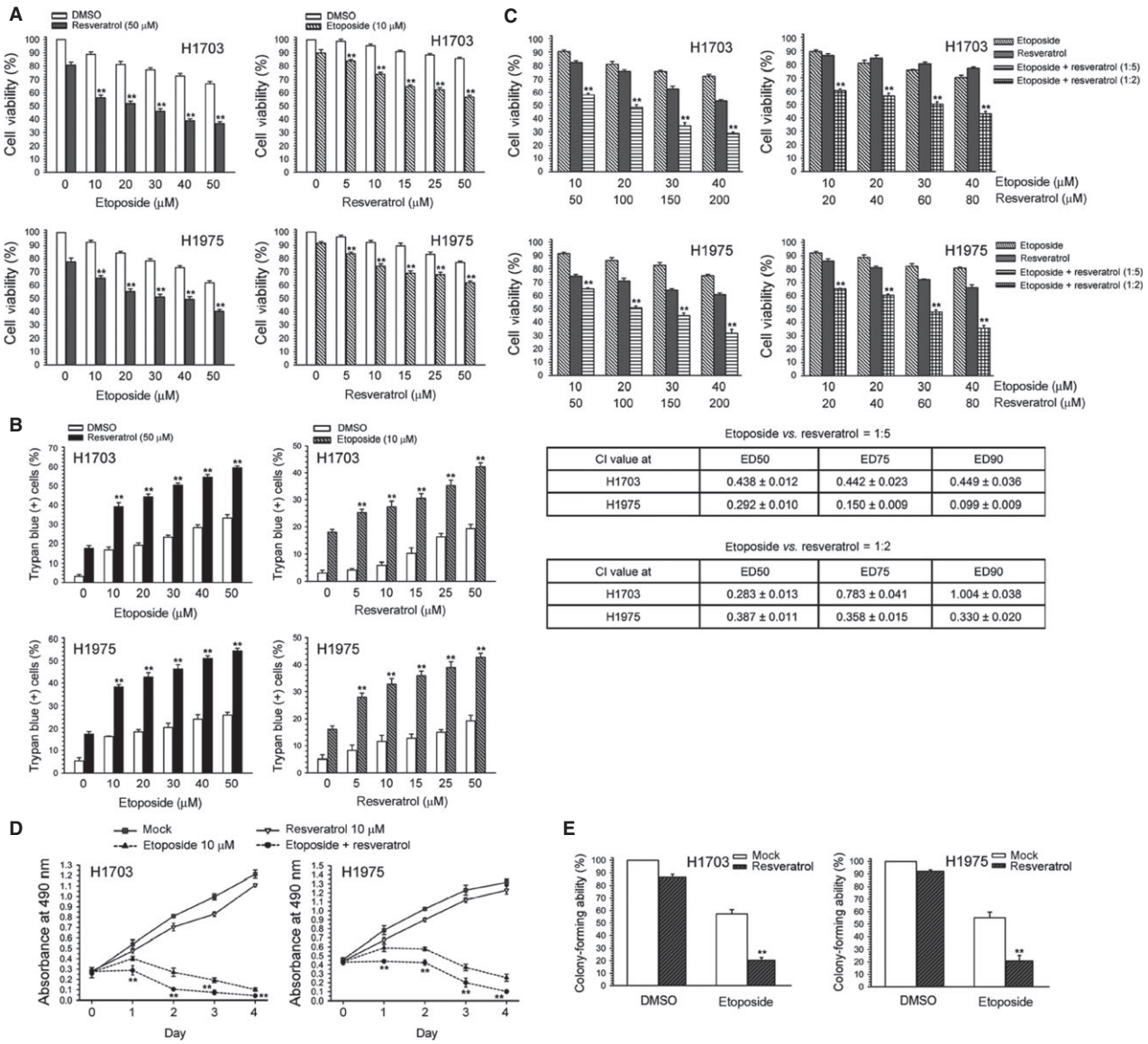
and H1975 cells. The results showed that combined etoposide and resveratrol had a synergistic cytotoxic effect on human NSCLC cells.

Resveratrol decreased etoposide-induced XRCC1 protein and mRNA expression in human lung cancer cells.

To assess the mechanism of the synergistic effects, we hypothesized that resveratrol would affect XRCC1 expression. To test this hypothesis, H1703 and H1975 cells were exposed to resveratrol (25, 50, 100, 150, 200 μM) and/or etoposide (10, 20, 40 μM) for 24 hr. In fig. 5A,B, resveratrol decreased XRCC1 mRNA and protein levels in H1703 and H1975 cells. As a result, XRCC1 protein and mRNA levels were induced by etoposide treatment in a dose-dependent manner, but inhibited by resveratrol treatment (fig. 5C,D). In addition, resveratrol suppressed the protein levels of phospho-ERK1/2, phospho-AKT in etoposide-exposed NSCLC cells (fig. 5D).

Transfection with AKT-CA or MKK1-CA vectors enhanced the XRCC1 protein level and the cell survival suppressed by resveratrol and etoposide.

We investigated whether resveratrol-mediated XRCC1 down-regulation was correlated with MKK1/2-ERK1/2 and PI3K-AKT down-regulation in etoposide and resveratrol-exposed NSCLC cells. Overexpression of MKK1-CA or AKT-CA could rescue the cellular XRCC1 protein and mRNA levels that were suppressed by resveratrol and etoposide (fig. 5E,F). Also, both MKK1-CA and AKT-CA vector transfection could rescue H1703 and H1975 cell viability after being decreased by resveratrol and etoposide (fig. 6A,B). Moreover, overexpression of MKK1-CA or AKT-CA could reverse the anti-proliferative effect of the two-drug combination (fig. 6C). Therefore, down-regulation of ERK1/2 and AKT-mediated XRCC1 expression by resveratrol enhanced the etoposide-induced cytotoxicity in H1703 and H1975 cells.



Etoposide *vs.* resveratrol = 1:5

CI value at	ED50	ED75	ED90
H1703	0.438 ± 0.012	0.442 ± 0.023	0.449 ± 0.036
H1975	0.292 ± 0.010	0.150 ± 0.009	0.099 ± 0.009

Etoposide *vs.* resveratrol = 1:2

CI value at	ED50	ED75	ED90
H1703	0.283 ± 0.013	0.783 ± 0.041	1.004 ± 0.038
H1975	0.387 ± 0.011	0.358 ± 0.015	0.330 ± 0.020

Fig. 4. Resveratrol co-treatment with etoposide synergistically enhanced cytotoxicity. (A, B) Left panel, etoposide (10, 20, 30, 40 and 50 μM) and/or resveratrol (50 μM) were added to H1703 or H1975 cells for 24 hr. Right panel, etoposide (10 μM) and/or resveratrol (5, 10, 15, 25 and 50 μM) were added to H1703 or H1975 cells for 24 hr. Cytotoxicity was determined by assessment with MTS (A) and trypan blue exclusion assay (B). (C) Etoposide and resveratrol were combined at a ratio of 1:5 or 1:2, and the MTS assay was used to analyse cell viability. The mean CI values at a fraction affected (FA) of 0.50, 0.75 and 0.90 for etoposide and resveratrol combined treatment were averaged for each experiment and used to calculate the mean between three independent experiments. (D) Cells were treated with etoposide (10 μM) and/or resveratrol (10 μM) for 1–4 days after which living cells were determined by MTS assay. $^{**}p < 0.01$ using Student's *t*-test for comparison between cells treated with a drug alone or with an etoposide/resveratrol combination. (E) Etoposide (0.1 μM) and/or resveratrol (10 μM) were added to cells for 24 hr, and cytotoxicity was determined by colony-forming ability assay. **Denotes $p < 0.01$, using Student's *t*-test for comparison between the cells treated with etoposide alone or etoposide/resveratrol combination.

Discussion

In this study, up-regulation of XRCC1 expression by etoposide was correlated with ERK1/2 and AKT activation in H1703 and H1975 cells. Inactivation of ERK1/2 or AKT activity by resveratrol could decrease XRCC1 mRNA and protein levels and cell survival in etoposide-exposed cells. Resveratrol is a small molecule, a natural component of grapes and red wine that has been proved be a potential anti-cancer and chemopreventive agent [13,21]. Its anti-cancer properties and low toxicity [22] make it an ideal candidate for use in combination with standard chemotherapy drugs. Resveratrol augmented the anti-proliferative effect of MMC on colorectal cancer cells [20]. Moreover, resveratrol-induced radiosensitization is associated with marked increases in ROS production, DNA double-strand breaks and senescence induction in irradiated NSCLC cells [23].

XRCC1 is a key mediator of SSB DNA repair in cells [24]. Down-regulation of XRCC1 expression resulted in decreased

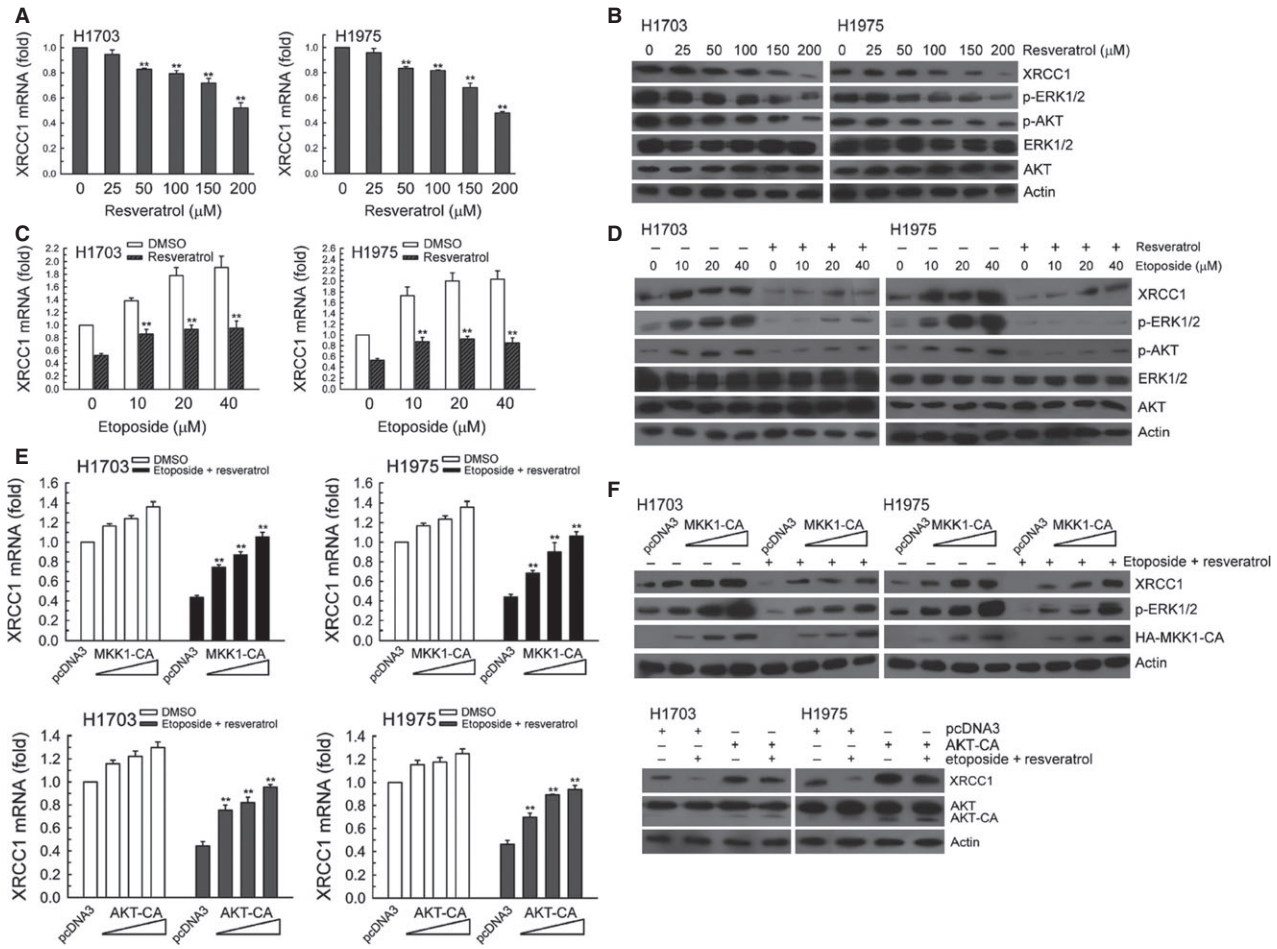


Fig. 5. Resveratrol decreased XRCC1 protein and mRNA levels in etoposide-exposed non-small-cell lung cancer (NSCLC) cells. (A and B) H1703 or H1975 cells (10^6) were cultured in complete medium for 18 hr and then were exposed to resveratrol for 24 hr. (C and D). Cells were cultured in complete medium for 18 hr and then were exposed to etoposide (10, 20, 40 μM) and resveratrol (100 μM) for 24 hr. After treatment as the above, total RNA was isolated and subjected to real-time polymerase chain reaction (PCR) for XRCC1 mRNA expression (A and C). The cell extracts were examined by Western blot for determination of XRCC1, phospho-ERK1/2, phospho-AKT, ERK1/2, AKT and actin protein levels (B and D). The means ± standard deviation (S.D.) from four independent experiments. **Denotes $p < 0.01$, respectively, using Student's *t*-test for comparison between the cells treated with etoposide/resveratrol alone or combined. (E and F) MKK1-CA (1, 3, 5 μg) or AKT-CA (5 μg) or pcDNA3 (5 μg) expression plasmids were transfected into cells using lipofectamine. After expression for 24 hr, the cells were treated with etoposide (10 μM) and resveratrol (50 μM) for an additional 24 hr, total RNA was isolated and subjected to real-time PCR for XRCC1 mRNA expression. The means ± standard deviation (S.D.) from four independent experiments. **Denotes $p < 0.01$, respectively, using Student's *t*-test to compare cells treated with etoposide and resveratrol in MKK1-CA or AKT-CA *versus* pcDNA3-transfected cells (E). The whole-cell extracts were collected for Western blot analysis (F).

SSB repair capacity and enhanced MMS-induced cytotoxicity in human breast cancer cell lines [11]. In this study, down-regulation of XRCC1 by resveratrol played a role in enhancing etoposide-induced cytotoxic effects in NSCLC cells. Consistent with previous studies, irinotecan, another chemotherapeutic agent to induce DNA damaging used to treat patients with advanced gastric cancer that had progressed on cisplatin, was found to inhibit the expression of XRCC1 effectively, and leading to an increase in the sensitivity of resistant cells to cisplatin [25]. Previous studies indicated that ionizing radiation-induced XRCC1 expression was described to be dependent on the EGFR/MAPK/ERK pathway [26–28]. Our present data showed that XRCC1 expression by etoposide was correlated with ERK1/2 and AKT activity. However, whether inactivation of ERK1/2 and AKT signals is involved in inhibiting the XRCC1-mediated DNA repair response requires further examination.

Taken together, we report first that resveratrol has a synergistic cytotoxic effect with etoposide in NSCLC cells through suppression of XRCC1. We suggest that decreasing XRCC1 expression could be exploited as a novel strategy to sensitize lung cancer cells to etoposide and enhance the therapeutic effect of etoposide in patients with NSCLC.

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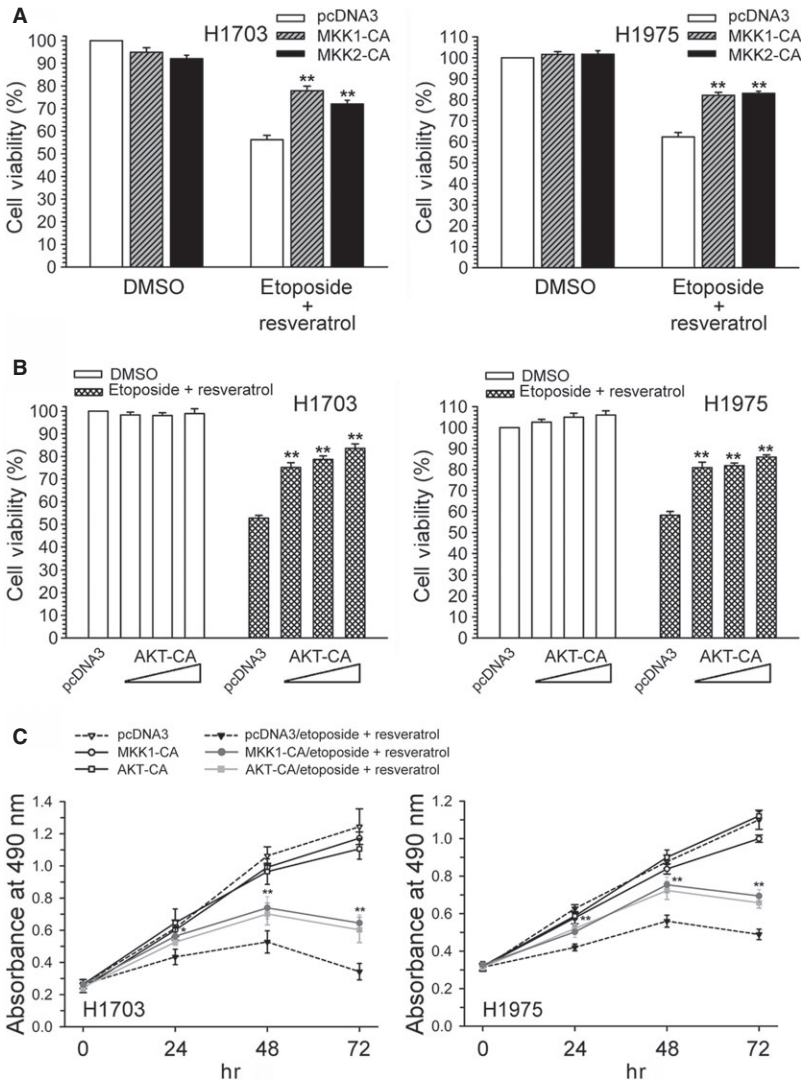

Fig. 6. Overexpression of AKT-CA or MKK1-CA reduced the resveratrol and etoposide-induced XRCC1 suppression and synergistic cytotoxic effects. (A and B) MKK1/2-CA (5 μg) or AKT-CA (1, 3, 5 μg) or pcDNA3 (5 μg) expression plasmids were transfected into cells using lipofectamine. After expression for 24 hr, the cells were treated with etoposide (10 μM) and resveratrol (50 μM) for an additional 24 hr. Cytotoxicity was determined by assessment with the MTS assay. (C) MKK1-CA (5 μg) or AKT-CA (5 μg) or pcDNA3 (5 μg) expression plasmids were transfected into cells using lipofectamine, and then the cells were treated with etoposide (10 μM) and/or resveratrol (50 μM) for 24, 48 or 72 hr after which living cells were determined by the MTS assay. $^{**}p < 0.01$, by Student's *t*-test to compare cells treated with etoposide and resveratrol in MKK1/2-CA or AKT-CA *versus* pcDNA3-transfected H1703 or H1975 cells.

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Conflict of Interest

None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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