Inhibition of p38 MAPK-Dependent Excision Repair Cross-Complementing 1 Expression Decreases the DNA Repair Capacity to Sensitize Lung Cancer Cells to Etoposide

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Abstract
Etoposide (VP-16), a topoisomerase II inhibitor, is an effective anticancer drug currently used for the treatment of a wide range of cancers. Excision repair cross-complementary 1 (ERCC1) is a key protein involved in the process of nucleotide excision repair. High level of ERCC1 expression in cancers is associated with resistance to DNA damage-based chemotherapy. In this study, the effects of p38 mitogen-activated protein kinase (MAPK) signal on the ERCC1 expression induced by etoposide in non–small cell lung cancer (NSCLC) cell lines was investigated. Etoposide increased phosphorylated MAPK kinase 3/6 (MKK3/6)-p38 MAPK and ERCC1 protein and mRNA levels in A549 and H1975 cells. Moreover, SB202190, a p38 inhibitor, or knockdown of p38 expression by specific short interfering RNA (siRNA) significantly decreased the etoposide-induced ERCC1 protein levels and DNA repair capacity in etoposide-exposed NSCLC cells. Enhancement of p38 activation by constitutively active MKK6 (MKK6E) increased ERCC1 protein levels. Specific inhibition of ERCC1 by siRNA significantly enhanced the etoposide-induced cytotoxicity and hypoxanthine guanine phosphoribosyltransferase (hprt) gene mutation rate. Moreover, the Hsp90 inhibitor 17-allylamino-17-demethoxygeldanamycin (17-AAG) could decrease the etoposide-induced p38 MAPK-mediated ERCC1 expression and augment the cytotoxic effect and growth inhibition by etoposide. 17-AAG and etoposide-induced synergistic cytotoxic effect and DNA repair capacity decrease could be abrogated in lung cancer cells with MKK6E or HA-p38 MAPK expression vector transfection. Our results suggest that in human NSCLC cells, ERCC1 is induced by etoposide through the p38 MAPK pathway, and this phenomenon is required for NSCLC survival and resistant DNA damage. Mol Cancer Ther; 11(3); 561–71. ©2011 AACR.

Introduction
Excision repair cross-complementary 1 (ERCC1) is one of the critical proteins within nucleotide excision repair (NER; ref. 1), and ERCC1-xeroderma pigmentosum F (ERCC1-XPF) catalyses incision on the incision 5' side to the site of DNA damage (2, 3). ERCC1 expression reflects DNA repair capacity and clinical resistance (4, 5). For example, Chinese hamster cell lines defective in ERCC1 show hypersensitivity to UV light and to DNA interstrand cross-linking agents (4, 6). The expression of ERCC1 is elevated in tissues from patients refractory to cisplatin therapy (7). Overexpression of the ercc1 gene is associated with a platinum-resistant phenotype in human non–small cell lung cancer (NSCLC; ref. 8), and the patients with ERCC1-negative tumors have a longer survival time than those with ERCC1-positive tumors in NSCLC (9). Etoposide is an epipodophyllotoxin used in the therapy of a wide spectrum of cancers (10). 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 (11). The first-line therapeutic option for patients with advanced NSCLC includes chemotherapy with a platinum-containing compound such as cisplatin or carboplatin in combination with cytotoxic agent such as etoposide (12–14). However, whether etoposide affects ERCC1 expression in NSCLC is unknown, and the role of ERCC1 in etoposide-induced cytotoxicity and mutagenicity has not been investigated.

Mitogen-activated protein kinase (MAPK) pathways such as p38 MAPK, extracellular-signal-regulated kinases...
(ERK), and c-Jun N-terminal kinases (JNK) are implicated in the response to chemotherapeutic drugs (15). MAPK pathway signaling modules consist of a 3-tiered kinase core where a MAPK kinase kinase activates a MAPK kinase (MKK), which in turn activates a MAPK (16). The primordial MAPK cascades are ubiquitously expressed and respond to various external cues and drugs. Previous studies have indicated that etoposide induces apoptosis in salivary gland acinar cells by activating JNK and suppressing the activation of ERK signaling pathways (17). However, inactivation of ERK1/2 in KB carcinoma cells did not affect the cytotoxic effects of etoposide (18). Whether p38 MAPK signals are involved in etoposide-induced cytotoxic effect and ERCC1 expression in NSCLC has not been investigated.

17-allylamino-17-demethoxygeldanamycin (17-AAG) acts by blocking the binding of ATP to the chaperone protein—Hsp90; this action results in the destabilization of the Hsp90 client protein complexes and subsequent proteasomal degradation of the client proteins (19, 20). p38-AAG significantly enhances the activity of etoposide in human colon cancer HCT116 cells (21). The molecular mechanism of 17-AAG enhancement of the etoposide-induced cytotoxic effect through modulation of p38 MAPK signaling in NSCLC has yet to be defined. Here, we show that etoposide activated p38 MAPK and increased ERCC1 expression in A549 and H1975 NSCLC cell lines. Knockdown of ERCC1 expression or inactivation of p38 MAPK activity by SB202190 or 17-AAG reduced the DNA repair capacity and cell viability in etoposide-treated human lung cancer cells. These results suggest that p38 MAPK signal plays a role in ERCC1 expression and thereby, increases DNA repair ability and cell viability of NSCLC cells treated with etoposide. Thus, the present study raises the possibility that p38-mediated ERCC1 expression may represent a new target for sensitization of NSCLC cells to etoposide.

Materials and Methods

Drugs and reagents
Etoposide, 17-AAG, and cycloheximide were purchased from Sigma Chemical. SB202190 were purchased from Calbiochem-Novabiochem. The specific phospho-p38 MAPK (Thr180/Tyr182) and phospho-MKK3 (Ser189)/MKK6 (Ser207) antibodies were purchased from Cell Signaling. Rabbit polyclonal antibodies against ERCC1 (FL-297; sc-10785), p38(C-20; sc-535), HA(F-7; sc-7392), MEK3N(20; sc-959), and Actin(I-19; sc-1616) were purchased from Santa Cruz Biotechnology.

Cell lines and culture
Human lung adenocarcinoma H1975 cells [CRL-5908; American Type Culture Collection (ATCC)] and human lung carcinoma A549 cells derived from human alveolar type 2 cells (CCL-185; ATCC) were purchased from ATCC. The cells were cultured at 37°C in a humidified atmosphere containing 5% CO2 in RPMI-1640 complete medium supplemented with sodium bicarbonate (2.2%, v/v), l-glutamine (0.03%, w/v), penicillin (100 units/mL), streptomycin (100 μg/mL), and fetal calf serum (10%). The cell lines were routinely tested to confirm that they were free of Mycoplasma. All experiments were done with exponentially growing cells. The cells were treated with drugs after a period of 12 hours for the cells to attach.

Western blot analysis
After different treatments, the cells were rinsed twice with cold PBS and lysed in whole cell extract buffer [20 mmol/L HEPES (pH 7.6), 75 mmol/L NaCl, 2.5 mmol/L MgCl2, 0.1 mmol/L EDTA, 0.1% Triton X-100, 0.1 mmol/L Na3VO4, 50 mmol/L NaF, 1 μg/mL leupeptin, 1 μg/mL aprotinin, 1 μg/mL pepstatin, and 1 mmol/L 4(2-aminoethoxy)benzenesulfonfluryl fluoride]. Equal amounts of proteins from each set of experiments were subjected to Western blot analysis as previously described (22). The relative protein blot intensities were determined using a computing densitometer equipped with the ImageQuant analysis program (Amersham Biosciences). The relative fold under each blot was calculated by averaging the results of 4 independent experiments and was normalized by arbitrarily setting the densitometry of control cells to 1.

Transfection with short interfering RNA or MKK6E and HA-p38 MAPK vectors
The sense-strand sequences of short interfering RNA (siRNA) duplexes were as follows: ERCC1: 5'-GGAGACUGCGCUAGAUGUGU-3', p38: 5'-GAACUCGGCUUACUUAAC-3', and scrambled (as a control): 5'-GGCCGCUUUGAGATCTG-3' (Dharmacon Research). Cells were transfected with siRNA duplexes (200 nmol/L) by using Lipofectamine 2000 (Invitrogen) for 24 hours. Plasmids transfection of MKK6E (a constitutively active form of MKK6) and HA-p38 MAPK were achieved as previously described (23, 24). Exponentially growing human lung cancer cells (106) were plated for 18 hours, and then MKK6E expression vectors were transfected into A549 or H1975 cells using Lipofectamine 2000 (Invitrogen).

Quantitative real-time PCR
PCRs were carried out 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'-GGGTGACTGAATGTCTGACCA-3'; GAPDH forward primer, 5'-CATGAGAAGTATGACAA-3'; GAPDH reverse primer, 5'-AGTCTTCAAGAAGGGGTC-3'. For each sample, the data were normalized to the housekeeping gene GAPDH.

Reverse transcription-PCR
RNA was isolated from cultured cells using TRIzol (Invitrogen) according to the manufacturer’s instructions.
Reverse transcription-PCR (RT-PCR) was conducted with 2 μg of total RNA using random hexamers following the Moloney murine leukemia virus reverse transcriptase cDNA synthesis system (Invitrogen). The final cDNA was used for subsequent PCRs. ERCC1 was amplified using primers with the sequence of 5'-CCCTGGGAATTTGGCGACGTAA-3' (forward) and 5'-CTCACAGTTTACATGTCC-3' (reverse) in conjunction with a thermal-cycling program consisting of 26 cycles of 95°C for 30 seconds, 61°C for 30 seconds, and 72°C for 60 seconds. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was amplified as an internal control. The GAPDH primers used for subsequent PCRs. ERCC1 was amplified using primers with the sequence of 5'-CCCTGGGAATTTGGCGACGTAA-3' (forward) and 5'-CTCACAGTTTACATGTCC-3' (reverse). Expression of GAPDH was used as a control to measure the integrity of the RNA samples.

Host cell reactivation assay
For host cell reactivation assays, the pGL4.13-Luc plasmid was damaged in vitro by either exposure to 200 J/m² UV-C for 30 minutes or treatment with 250 nmol/L cisplatin at 37°C for 1 hour 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 hours 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). To investigate whether the ERCC1 was involved for the modulation of DNA repair in etoposide exposed cells, cells were cotransfected with either cisplatin damaged or UV-irradiated pGL4.13-Luc in the presence of si-ERCC1 RNA or control siRNA.

 Luciferase assay
Cells were grown at 10⁴ cells per well in 96-well plates and were transfected with 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 carried out 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 according to the readings of mean ± SEM of luminescence. 

Cell viability assay
In vitro 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) was added, the cells were incubated for a further 2 hours, and the absorbance was measured at 490 nm using an ELISA plate reader (Biorad Technologies).

Colony-forming ability assay
Immediately after the drugs treatment, cells were washed with PBS and trypsinized for the determination of cell numbers. The cells were plated at a density of 500 to 1,000 cells on a 60 mm-diameter Petri dish in triplicate for each treatment. The cells were cultured for 10 to 14 days, and 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.

Mutagenicity assay
The etoposide-treated or untreated cells were maintained in exponential growth for 7 days to allow for the expression of resistance to 6-thioguanine (6-TG). One to 2 million cells from each treatment were plated onto 100 mm-Petri dishes (10 dishes total) in a selective medium containing 40 μmol/L of 6-TG, followed by incubation for 14 days. The plating efficiency of cells at the time of selection was also assayed in a nonselective medium to correct the observed mutant frequency. The mutant frequency was calculated to be the total number of 6-TG-resistant colonies divided by the total number of clonable cells at selection time (25).

Combination index analysis
The cytotoxicity induced by the combined treatment with etoposide and 17-AAG 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 CI analysis was carried out using CalcuSyn software (Biosoft). 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 3 independent experiments.

Trypan blue dye exclusion assay
Cells were treated with etoposide and/or 17-AAG for 24 hours. Trypan blue dye can be excluded from living cells, but can penetrate into dead cells. The proportion of dead cells was determined by hemocytometer to count the number of cells stained with trypan blue.

Statistical analyses
For each protocol, 3 or 4 independent experiments were carried out. Results were expressed as the mean ± SEM. Statistical calculations were carried out by using Sigma-Plot 2000 (Systat Software). Differences in measured variables between experimental and control groups were assessed by unpaired t test. P < 0.05 was considered statistically significant.
**Results**

**Etoposide increases levels of phospho-MKK3/6-p38 MAPK and ERCC1 protein and mRNA in a time- and dose-dependent manner**

We first examined the effect of etoposide on the activation of MKK3/6-p38 MAPK in NSCLC cell lines. In Fig. 1, treatment of A549 and H1975 cells with etoposide induced an increase in the phosphorylation of MKK3/6-p38 MAPK in a time- and dose-dependent manner (Fig. 1A and B). Interestingly, the results in Fig. 1A and B also showed that etoposide induced ERCC1 protein expression; this was accompanied by an increase in phospho-MKK3/6-p38 MAPK protein levels in A549 and H1975 cells. To elucidate whether the observed etoposide stimulation of ERCC1 protein expression occurred at the transcriptional level, various concentrations of etoposide were added to A549 or H1975 cells for 4 to 24 hours, and total RNA was isolated and subjected to real-time PCR analysis. As shown in Fig. 1C and D, etoposide also increased endogenous ercc1 mRNA level in human lung cancer cells.

**Upregulation of ERCC1 by etoposide in a p38 MAPK activation manner**

To examine the role of p38 MAPK in the upregulation of ERCC1 by etoposide, we used a p38 specific inhibitor and specific siRNA duplexes. As shown in Fig. 2A and B, p38 MAPK inhibitor, SB202190, treatment decreased ERCC1 protein and mRNA levels in etoposide-exposed A549 or H1975 cells. Moreover, silencing of p38 MAPK significantly led to a decrease in the ERCC1 protein expression by etoposide (Fig. 2C). In contrast, overexpressing the HA-p38 MAPK vector exhibited a higher phospho-p38 and enhanced ERCC1 expression in response to etoposide as compared with the control cells (Fig. 2D). Furthermore, A549 or H1975 cells were transiently transfected with a plasmid carrying MKK6E, a constitutively active form of MKK6. Transfection with MKK6E increased cellular p38 MAPK phosphorylation and ERCC1 protein expression compared with transfection with the control vector—pcDNA3 (Fig. 2D). Therefore, we concluded that etoposide increased ERCC1 expression in a p38 MAPK activation manner.

**Etoposide induces activation of p38 MAPK that contributes to increases ERCC1 protein and mRNA stability**

Furthermore, we examined the possible mechanisms for posttranscriptional regulation of ercc1 transcripts under etoposide treatment. To evaluate the stability of ercc1 mRNA in etoposide-exposed A549 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 and RT-PCR at 4, 8, and 12 hours after treatment. As shown in Supplementary Fig. S1A and B, after actinomycin D coexposure for 12 hours, etoposide treatment showed higher levels of ercc1 mRNA relative to untreated cells. To investigate whether the ERCC1 protein expression affected by treatment with etoposide was regulated at the posttranslational level, cycloheximide (an inhibitor of de novo protein synthesis) was added with etoposide.
for 4, 8, and 12 hours. ERCC1 protein levels were progressively reduced with time in the presence of cycloheximide (Supplementary Fig. S1C). However, etoposide treatment significantly prevented ERCC1 degradation after cycloheximide treatment compared with untreated cells (Supplementary Fig. S1C). Therefore, ERCC1 protein was more stable after etoposide treatment in A549 and H1975 cells. Interestingly, blocking p38 MAPK activation by SB2020190 suppressed etoposide-induced ERCC1 mRNA and protein stability (Supplementary Fig. S1). This result indicated that etoposide increased ERCC1 protein and mRNA levels through the improvement of protein and mRNA stability of ERCC1 in a p38 MAPK activation manner.

**Downregulation of ERCC1 decreases the DNA repair capacity in etoposide-exposed NSCLC cells**

To investigate whether p38 MAPK-mediated ERCC1 expression 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 or UV. The pGL4.13-Luc reporter plasmid was treated with 250 nmol/L cisplatin or with 200 J/m² UV-C. NSCLC cells were transfected with cisplatin-treated or UV-irradiated pGL4.13-Luc plasmid and then coadded with etoposide and SB202190. As shown in Fig. 3A, inhibition of p38 MAPK activation by SB202190 was less capable of generating luciferase activity from the cisplatin-treated or UV-irradiated vector in etoposide-treated A549 cells. Consistent with above result, silencing of p38 MAPK expression by specific siRNA also could decrease DNA repair capacity in etoposide-exposed A549 and H1975 cells (data not shown). In contrast, overexpressing HA-p38 MAPK could slightly increase the DNA repair capability in etoposide-treated A549 cells (Supplementary Fig. S2A and B). The damaged plasmid did not affect ERCC1 levels under A549 cells were treated with or without etoposide (Supplementary Fig. S2C). Next, we used si-ERCC1 RNA to knock down ERCC1 expression in etoposide-treated A549 cells, and then analyzed the DNA repair capacity as above. ERCC1 knockdown led to a decrease in ERCC1 protein expression (Supplementary Fig. S2D) and reduced the capability of generating luciferase activity of the cisplatin-treated or UV-irradiated vector in etoposide-treated A549 cells (Fig. 3B).
Furthermore, a mutagenicity assay showed that suppression of ERCC1 protein expression significantly increased the etoposide-induced hypoxanthine-guanine phosphoribosyltransferase (hprt) gene mutation frequency (Fig. 3C). In addition, the hprt gene mutation in si-ERCC1 RNA-transfected cells alone was slightly higher than in controls ($3.95 \times 10^{-6}$ compared with $1.69 \times 10^{-6}$, respectively; Fig. 3C). Taken together, the results reflected that the downregulation of etoposide-induced p38 MAPK-mediated ERCC1 expression could decrease the cellular ability to repair the DNA damage in etoposide-exposed human NSCLC cells.

Blocking p38 MAPK activation or knockdown of ERCC1 sensitizes NSCLC cells to etoposide

Furthermore, to verify the involvement of ERCC1 in the cytotoxicity induced by etoposide, ERCC1 protein expression was knocked down using specific siRNA duplexes. In Fig. 4A, ERCC1 siRNA resulted in inhibition of the cellular and etoposide-induced ERCC1 expression in A549 or H1975 cells. However, knockdown of ERCC1 expression had no effect on p38 MAPK activation by etoposide. Figure 4B showed that etoposide treatment decreased cell viability in a dose-dependent manner as determined by the MTS and
colony-forming ability assay. Suppression of ERCC1 protein expression by si-ERCC1 RNA resulted in increased sensitivity to etoposide as compared with si-control transfected cells (Fig. 4B). In addition, more inhibition of cell growth was induced by the combination of ERCC1 siRNA and etoposide than etoposide alone in NSCLC cells as shown in Fig. 4C. Furthermore, to examine the role of the p38 MAPK in the cytotoxic effect of etoposide, we used the p38 inhibitor SB202190 or si-p38 RNA to block etoposide-induced p38 MAPK activation. Treatment of the cells with the specific inhibitors or knockdown of p38 MAPK expression abolished the phosphorylation of the p38 MAPK in response to etoposide (data not shown). As shown in Fig. 4D, SB202190 cotreatment or p38 MAPK silencing significantly further decreased cell viability in etoposide-exposed A549 or H1975 cells compared with etoposide treatment alone. In contrast, MKK6E transfection could increase cell viability that was decreased by etoposide (Supplementary Fig. S3). Taken together, ERCC1-siRNA and downregulation of p38 MAPK activation by p38 MAPK-siRNA or pharmacologic inhibitor SB202190 were both capable of enhancing etoposide sensitivity in NSCLC.

17-AAG decreases etoposide-elicited phosphorylated p38 MAPK and ERCC1 protein and mRNA levels

Previous studies have indicated that Hsp90 inhibitor 17-AAG treatment significantly suppressed the lipopolysaccharide-induced p38 MAPK activity (26). In addition, 17-AAG significantly enhanced the activity of etoposide in human colon cancer HCT116 cells (21). Thus, we proposed that 17-AAG could enhance etoposide-induced cytotoxic effect through the downregulation of p38 MAPK-mediated ERCC1 expression in NSCLC cells. First, the human NSCLC cell lines A549 and H1975 were exposed to various concentrations of etoposide (10, 20, and 40 μmol/L) and 17-AAG (1 μmol/L) for 24 hours. In Supplementary Fig. S4A, 17-AAG treatment resulted in inhibiting etoposide-induced p38 and ERCC1 protein and mRNA levels. Moreover, the results from real-time PCR analysis showed that 17-AAG decreased ercc1 mRNA level.
under etoposide exposure in A549 and H1975 cells (Supplementary Fig. S4B).

Combined use of etoposide with 17-AAG causes synergistic cytotoxic effect and growth inhibition in NSCLC cells

Second, we investigated the effect of the combination of 17-AAG and etoposide on cell viability using MTS and trypan blue exclusion assays. For these studies, cells were treated with 17-AAG (0.1–1 μmol/L), etoposide (10–40 μmol/L), or the 2-drug combination for 24 hours. We found that treatment with 17-AAG plus etoposide for 24 hours resulted in greater loss of cell viability than that caused by either 17-AAG or etoposide alone in A549 and H1975 cells (Fig. 5A and B). Likewise, CI values for etoposide and 17-AAG were less than 1, indicating synergism (Fig. 5C). In addition, A549 and H1975 cells were exposed to 17-AAG and/or etoposide, and cell proliferation was determined over 1 to 4 days after exposure to the drugs. As shown in Fig. 5D, the combination of 17-AAG and etoposide more effectively inhibited cell growth than either treatment alone. We concluded that 17-AAG could sensitize human lung tumor cells to etoposide and enhance the etoposide-elicited growth inhibition effect.

17-AAG and etoposide-induced synergistic cytotoxic effect and DNA repair capacity decrease can be abrogated in lung cancer cells with MKK6E or HA-p38 MAPK expression vector transfection

We then explored the roles of p38 MAPK pathway directly affected by 17-AAG in the cellular response to etoposide. A549 or H1975 cells were transfected with MKK6E plasmids, then treated with 17-AAG and etoposide, and finally assessed using the MTS assay. Transfection with MKK6E could enhance ERCC1 expression and cell survival, which was suppressed by cotreatment with 17-AAG and etoposide (Fig. 6A and B). On the other hand, the p38 MAPK inhibitor SB202190 markedly enhanced the downregulation of ERCC1 and cytotoxicity induced by etoposide and 17-AAG in A549 and H1975 cells (Fig. 6C and D). As an end result, the combination of etoposide and 17-AAG resulted in less repair capacity for
cisplatin-damaged DNA as compared with control cells (Supplementary Fig. S5). However, transfection with MKK6E or HA-p38 MAPK expression could restore the DNA repair capacity, which was reduced by 17-AAG and etoposide (Supplementary Fig. S5). These results indicated that 17-AAG inhibited the p38 MAPK-mediated ERCC1 expression and DNA repair capacity in NSCLC cells and consequently, increased etoposide-induced cytotoxicity.

Discussion

In this study, our results illustrated for the first time that etoposide-induced p38 MAPK activation participated in increasing the ERCC1-mediated DNA repair capacity and cell survival in NSCLC cells. Downregulation of p38 MAPK activation by specific p38 MAPK inhibitor or siRNA could enhance the sensitivity to etoposide of NSCLC. Previous studies indicated that inhibition of p38 activation by SB203580, a specific inhibitor of p38α and p38β, augments apoptosis of lymphoma cells treated with etoposide (27). Etoposide induces the downregulation of kinase phosphatase-1 (MKP-1), which correlates with persistent phosphorylation of ERK1/2, resulting in cell apoptosis (28). Etoposide induces JNKs activation and triggers apoptosis in endometrial cancer cells (29).

MAPK pathways have been reported to be involved in NER in a number of studies. In human hepatoma cells, epidermal growth factor (EGF)-mediated ERCC1 induction is dependent on the MKK-ERK1/2 pathway and involves the transcriptional factor GATA-1 (30). However, the use of SB203580 and SP600125 (JNKs inhibitor) to block the p38 and JNK pathways, respectively, did not prevent ERCC1 induction by EGF (30). Furthermore, in NIH3T3 and MCF-7 cells, a marked increase of ERCC1 by the activated H-Ras through the upregulation of AP-1 transcriptional activity has been reported (31). In addition, cisplatin treatment in human ovarian cancer A2780/CP70 cells led to an increase in ercc1 mRNA expression, and ercc1 mRNA induction by cisplatin is transcriptionally regulated by decreasing MZF1 repressor and increasing AP1 binding to the ERCC1 promoter (32, 33). Activation of JNK upon UV enhances XPF mRNA and protein levels and stimulates NER in mouse fibroblast (34). Moreover, blocking JNK activity sensitizes the tumor cells to cisplatin through modulating NER (35, 36). A previous study indicated that p38 MAPK regulates damaged DNA-binding protein 2 (DDB2) degradation for facilitating NER factor assembly (37). Recently, a study indicated that the loss of tumor suppressor phosphatase and tensin homolog suppresses the expression of xeroderma pigmentosum C through upregulating AKT-dependent p38 signaling inhibition in skin papilloma and squamous cell carcinoma (38). In this study, p38 MAPK influenced the repair outcome through its effect on ERCC1 expression, which is required for the repair etoposide-induced DNA damage in NSCLC cells.

Curcumin sensitizes glioma cells to etoposide, and the effects correlate with reduced expression of DNA repair enzymes (ERCC-1, O-6-methylguanine-DNA methyltransferase, DNA-dependent protein kinase, Ku70, and Ku80; ref. 39). In this study, we found that 17-AAG
enhanced the etoposide-induced growth inhibitory and cytotoxic effects in human NSCLC cells by inhibition of the MKK3/6-p38 MAPK signaling pathway, which mediated the expression of ERCC1. To our knowledge, our results firstly showed that the inhibition by 17-AAG of MKK3/6-p38 MAPK-ERCC1 pathways in NSCLC lines exerts synergistic effects on etoposide-induced cell death. Cancer cells contain elevated levels of active Hsp90, which is a chaperone required for the posttranslational stability of its protein substrates or client proteins (40). 17-AAG, a geldanamycin derivative (41), binds to a conserved ATP interaction pocket in the Hsp90 NH2-terminal domain (42) and has activity against a variety of human cancers (43, 44). Inhibition of Hsp90 results in the simultaneous blockade of multiple oncogenic signaling cascades, and sensitizes cancer cells to chemotherapeutic agents (45, 46). Consistently with our study, etoposide and 17-AAG had synergistic inhibitory effects on leukemia cells (47). Hence, Hsp90 activity inhibition and topoisomerase II poison warrants evaluation as a novel therapeutic strategy in NSCLC.

Tumor DNA repair capacity is frequently increased as a mechanism to evade cell death following chemotherapeutic drugs. In conclusion, our work is the first identification of ERCC1 induction by etoposide through the p38 MAPK pathway, and this phenomenon is required for NSCLC survival and resistant DNA damage. Combination treatment with an Hsp90 inhibitor 17-AAG significantly decreased the expression of ERCC1 and DNA repair capacity that are associated with enhancing chemosensitivity to etoposide in NSCLC cells. These results provide the way to designing rational combination therapies using Hsp90 inhibitors and topoisomerase II poisons for NSCLC therapy in the future.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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