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Title Page

Synergistic effect of curcumin and cisplatin via downregulation of thymidine phosphorylase and ERCC1

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Running Title Page

Running title: TP and ERCC1 regulated by curcumin enhances cisplatin sensitivity

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ABSTRACT

Curcumin (diferuloylmethane), a phenolic compound obtained from the rhizome of

Curcuma longa, is known to have antiproliferative and antitumor properties.

Thymidine phosphorylase (TP), an enzyme of the pyrimidine salvage pathway, is

considered an attractive therapeutic target and its expression could suppress cancer

cell death induced by DNA damage agents. Excision repair cross-complementary 1

(ERCC1) is a protein involved the process of nucleotide excision repair. The ERCC1

gene is expressed at high levels in cancers and has been associated with resistance to

platinum-based chemotherapy. In this study, the effects of curcumin on TP and

ERCC1 expression induced by cisplatin in non-small cell lung cancer (NSCLC) cell

lines was investigated. Exposure of the NSCLC cells to various concentrations of

curcumin (5–40 μM) downregulates the mRNA and protein levels of TP and ERCC1

through destabilization of the mRNA and proteins via a mechanism involving

inactivation of MKK1/2-ERK1/2. Depletion of endogenous TP or ERCC1 expression

by transfection with specific small interfering RNAs significantly decreases cell

viability in curcumin-exposed NSCLC cells. Curcumin enhances the sensitivity of

cisplatin treatment for NSCLC through inactivation of ERK1/2 and by decreasing the

TP and ERCC1 protein levels. Enhancement of ERK1/2 signaling by constitutively

active MKK1/2 (MKK1/2-CA) causes an increase in TP and ERCC1 protein levels

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and promotes cell viability after cotreatment with curcumin and cisplatin.

Enhancement of the cytotoxicity to cisplatin by administration of curcumin is

mediated by downregulation of the expression levels of TP and ERCC1 and by

inactivation of ERK1/2.

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Introduction

Lung cancer is a leading global cause of morbidity and mortality (Jemal et al., 2006).

Cisplatin-based chemotherapy has been widely used for the treatment of non-small

cell lung cancer (NSCLC). Cisplatin exerts cytotoxic effects through the formation of

covalent adducts and interferes with DNA replication, leading to cell death (Furuta et

al., 2002). However, the efficacy of this chemotherapy is frequently attenuated owing

to drug resistance in the advanced stage of NSCLC (Seve and Dumontet, 2005). The

mechanisms of cisplatin resistance have been proposed, including enhancement of

DNA repair and intracellular antioxidant activity, which block the induction of

apoptosis (Rosell et al., 2002; Rudin et al., 2003). This leads to overproduction of

pro-inflammatory mediators triggered by cancer cells and decreased drug

accumulation (Ohmichi et al., 2005; Siddik, 2003). As a result of these mechanisms,

there is increased expression of excision repair cross-complementation 1 (ERCC1), a

structure-specific DNA repair endonuclease responsible for incision at the 5′ site of

damaged DNA (Niedernhofer et al., 2004). This increased expression of ERCC1 in

cancer cells has been associated with more efficient removal of DNA adducts induced

by cisplatin, leading to clinical drug resistance (Altaha et al., 2004). Therefore, in

NSCLC, treatment with adjuvant cisplatin-based chemotherapy significantly

prolonged the survival rate among patients with ERCC1-negative tumors, relative to

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ERCC1-positive patients (Azuma et al., 2007).

Curcumin (diferuloylmethane), a well-known chemopreventive agent, has been reported to possess antioxidant, anti-inflammatory, antiproliferative, and anticarcinogenic activities in several different cancers (Shishodia et al., 2005). It has been reported that among the multiple biological effects of curcumin is an ability to suppress several intracellular signaling molecules, including NF-κB, and AP-1 as well as the members of the MAPK family signaling pathways (Chen and Tan, 1998; Han et al., 2002). The sensitizing effect of curcumin on cancer cell apoptosis has been reported for a variety of chemotherapeutic agents (Du et al., 2006; Weir et al., 2007) that are related to inactivation of NF-κB, modulation of p38 MAPK and AKT, and upregulation of death receptor 5 (Deeb et al., 2004; Jung et al., 2005; Weir et al., 2007). However, it is unclear whether curcumin could enhance the sensitivity of cisplatin through modulation of MKK1/2-ERK1/2 signaling in NSCLC.

Thymidine phosphorylase (TP) is an enzyme of the pyrimidine salvage pathway which is upregulated in cancers. TP expression is correlated with reduced apoptosis induced by DNA damage agents, microtubule-interfering agents, hypoxia, and Fas ligands (Ikeda et al., 2003; Jeung et al., 2005; Mori et al., 2002). Elevated levels of TP are associated with poor prognosis and shorter patient survival (Mori et al., 2000). However, it should be explored whether curcumin has the ability to regulate TP

expression, and the detailed molecular mechanism involved in this process should be characterized.

Curcumin has no adverse health effects and is therefore an excellent candidate for development of potential therapeutic strategies for treatment of cancer. In the present study, we have investigated the molecular mechanisms of curcumin alone and in combination with cisplatin for generation of cytotoxic effects in three different NSCLC cell lines. The evidence indicates that by downregulating TP and ERCC1 expression and inactivation of MKK1/2-ERK1/2 signaling in NSCLC cell lines, curcumin exerts a cytotoxic effect that is synergistic with the effect of cisplatin.

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Materials and Methods

Cell lines and reagents. Human lung bronchioloalveolar adenocarcinoma A549, lung squamous cell carcinoma H520, and lung carcinoma H1975 cell lines were obtained from the American Type Culture Collection (Manassas, VA), and 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 fetal calf serum (10%).

Curcumin, cisplatin, cycloheximide, and actinomycin D were obtained from Sigma (St Louis, MO). MG132, lactacystin, and U0126 were purchased from Calbiochem-Novabiochem (San Diego, CA, USA). Actinomycin D, MG132, lactacystin, and U0126 were dissolved in dimethyl sulfoxide (DMSO). Cycloheximide was dissolved in Milli-Q-purified water (Millipore, Billerica, MA, USA).

Western blot analysis. After the different treatments, equal amounts of protein from each set of experiments were subjected to western blot analysis as previously described (Ko et al., 2010). The specific phospho-ERK1/2 (Thr202/Tyr204) (#9101) and phospho-MKK1/2 (Ser217/Ser221) (#9121), antibodies were purchased from Cell Signaling (Beverly, MA, USA). The PD-ECGF(PGF-44C) (#sc-47702), ERCC1(FL-297) (#sc-10785), ubiquitin(P4D1) (#sc-8017), ERK2(K-23) (#sc-153),

HA(F-7) (#sc-7392), and Actin(I-19) (#sc-1616) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

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 three independent experiments and was normalized by arbitrarily setting the densitometry of control cells to 1.

Immunoprecipitation. After treatment, equal amount of proteins were immunoprecipitated using an anti-ubiquitin antibody and collected with protein G-Sepharose beads (GE Healthcare). The immunoprecipitate was then washed three times with cold lysis buffer and subjected to immunoblot analysis.

Small interfering RNA (si-RNA) transfection. The sense-strand sequences of siRNA used were as follows: TP: 5′-AUAGACUCCAGCUUAUCCA-3′; ERCC1: 5′-GGAGCUGGCUAAGAUGUGU-3′, and scrambled (as a control): 5′-GCGCGCUUUGUAGGATTCG-3′ (Dharmacon Research, Lafayette, CO). Cells were transfected with siRNA duplexes (200 nM) with Lipofectamine 2000 (Invitrogen) for 24 h.

Plasmid and Transfection. Plasmid transfection of MKK1-CA (a constitutively active form of MKK1, ΔN3/S218E/S222D) and MKK2-CA (a constitutively active

form of MKK2, ΔN4/S222E/S226D) was achieved as previously described (Ko et al., 2010). Exponentially growing human lung cancer cells (10^6) were plated for 18 h and the MKK1/2-CA expression vectors were transfected into cells using Lipofectamine (Invitrogen) before curcumin and cisplatin treatment.

Reverse Transcription-PCR. Total RNA was extracted from drugs treated NSCLC cells with TRIzol reagent (Invitrogen, Carlsbad, CA). cDNA was synthesized by using random hexamers following the Moloney murine leukemia virus reverse transcriptase cDNA synthesis system (Invitrogen). The final cDNA was used for subsequent PCRs. TP was amplified by using the primers with the sequence 5'-GGCATGGATCTGGAGGAGAC-3' (forward) and 5'-CTCTGACCCACGATACAGCA-3' (reverse) in conjunction with a thermal cycling program consisting of 40 cycles of 95°C for 30 s, 59°C for 30 s, and 72°C for 30 s. ERCC1 was amplified using primers with the sequence of 5′-CCCTGGGAATTTGGCGACGTAA-3′ (forward) and 5′-CTCCAGGTACCGCCCAGCTTCC-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

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GAPDH was used as a control to measure the integrity of the RNA samples.

Quantitative real-time PCR. 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: TP forward primer, 5'-

AGCTGGAGTCTATTCCTGGATT -3'; TP reverse primer, 5'-

GGCTGCATATAGGATTCCGTC -3'; ERCC1 forward primer, 5'-

GGGTGACTGAATGTCTGACCA -3', ERCC1 reverse primer, 5'-

GGGTACTTTCAAGAAGGGCTC -3'; GAPDH forward primer, 5'-

CATGAGAAGTATGACAACAGCCT -3'; GAPDH reverse primer, 5'-

AGTCCTTCCACGATACCAAAGT -3'. For each sample, the data were normalized

to the housekeeping gene GAPDH.

Measurement of cell viability. *In vitro* MTS assay was carried out essentially

according to the procedure described (Ko et al., 2010). The principle of this

convenient method is based on the conversion of tetrazolium salt (MTS) into a

chromatic, soluble formazan by a mitochondria enzyme, NAD-dependent

dehydrogenase, in live cells. Briefly, 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

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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 ELISA plate reader (Biorad Technologies, Hercules, CA).

Combination index analysis. The cytotoxicity induced by the combined treatment with curcumin and cisplatin 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 and 0.75 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 the drugs treatment, cells were washed with phosphate-buffered saline and trypsinized for the determination of cell numbers. The cells were plated at a density of 500 cells on a 60 mm-diameter Petri dish in triplicate for each treatment. The cells were cultured for 10–14 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.

Trypan blue dye exclusion assay. Cells were treated with curcumin and/or cisplatin

for 24 h. In each preparation, the cell viability of lung cancer cells was determined by the trypan blue dye exclusion assay; this dye was excluded by living cells and only penetrated the cell membrane of dead cells. The proportion of dead cells was determined by using a hemocytometer to count the number of stained cells.

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

Results

TP and ERCC1 protein and mRNA levels are decreased after curcumin exposure.

To evaluate the role of TP and ERCC1 in regulating the curcumin-induced cytotoxicity in NSCLC cell lines, A549, H520, and H1975 cells were treated with concentrations of curcumin ranging from 5 to 40 μM for 24 h and then examined for expression of TP and ERCC1 by western blot analysis. Fig. 1A demonstrated that curcumin significantly decreased the cellular TP and ERCC1 protein levels. Actin controls showed that the levels of actin protein were unaffected under these conditions. Moreover, western blot analysis using phosphospecific antibodies indicated a decrease of MKK1/2-ERK1/2 phosphorylation after curcumin treatment of the three cell lines (Fig. 1A). No differences in levels of endogenous unphosphorylated ERK1/2 were observed. To elucidate the mechanisms responsible for the elevated TP and ERCC1 expression levels observed in NSCLC cell lines, we first confirmed the expression of TP and ERCC1 mRNA by reverse transcription-PCR (RT-PCR) and real-time PCR. As shown in Figs. 1B and C, curcumin significantly and dose-dependently reduced the steady-state levels of TP and ERCC1 mRNA in NSCLC cell lines.

Curcumin treatment downregulates the protein and mRNA stability of TP and ERCC1 in NSCLC cell lines. Next, we examined the possible mechanisms for

posttranscriptional regulation of TP and ERCC1 transcripts under curcumin treatment.

To evaluate the mRNA stability of TP and ERCC1 in curcumin-exposed lung cancer cells, we treated these cells with curcumin (10 μM) for 9 h in the presence of actinomycin D to block *de novo* RNA synthesis for 3, 6, or 9 h and then measured the levels of the existing TP and ERCC1 mRNA by RT-PCR. As shown in Fig. 2A, curcumin treatment produced lower levels of TP and ERCC1 mRNA after cotreatment with actinomycin D relative to control cells. This indicates that curcumin decreases the stability of TP and ERCC1 mRNAs.

Furthermore, we investigated the mechanism of modulation of protein levels of TP and ERCC1 by curcumin by exploring the possibility that curcumin enhances the rate of TP and ERCC1 protein degradation. These cells were treated with curcumin for 9 h, followed by the addition of cycloheximide (an inhibitor of *de novo* protein synthesis) for 3, 6, and 9 h to evaluate the rate of degradation of the TP and ERCC1 proteins. In Fig. 2B, it can be seen that upon treatment of NSCLC cell lines with curcumin, the rates of degradation of TP and ERCC1 proteins were markedly increased relative to the untreated cells. The curcumin-mediated downregulation of the TP and ERCC1 mRNA and protein expression occurs through a decrease in the stabilities of the mRNAs and proteins.

Lactacystin and MG132 block the curcumin-induced downregulation of TP and

ERCC1 protein. To investigate whether the 26S proteasome is involved in the curcumin-induced degradation of TP and ERCC1 proteins, the 26S proteasome inhibitors lactacystin or MG132 was added with curcumin to the NSCLC cells. As shown in Fig. 2C, both lactacystin and MG132 prevented the curcumin-induced downregulation of TP and ERCC1. In addition, we examined the levels of ubiquitin conjugates on TP and ERCC1. In Fig. 2D, curcumin treatment increased the levels of ubiquitin-conjugated TP and ERCC1 in NSCLC cell lines. These results indicate that curcumin decreases the levels of TP and ERCC1 protein via the ubiquitin-mediated 26S proteasome degradation pathway in NSCLC cells.

ERK1/2 inactivation is correlated with decreased expression of TP and ERCC1 by curcumin. Next, we determined whether the MKK1/2–ERK1/2 signaling pathway inactivation is involved in the downregulation of TP and ERCC1 protein expression in curcumin-exposed NSCLC cells. The NSCLC cells were transfected with MKK1-CA (constitutive active MKK1) vectors, and then treated with curcumin (10–40 μM) for 24 h. As shown in Fig. 3A, the phosphorylation levels of ERK1/2 were higher in MKK1-CA–transfected A549 and H1975 cells than in pcDNA3-transfected cells. It was found that MKK1-CA transfection could also restore the phosphorylated ERK1/2, TP, and ERCC1 protein levels, which were decreased by curcumin treatment (Fig. 3A). Moreover, MKK1/2-CA was also found to be able to rescue the TP mRNA

downregulation by curcumin, as determined by RT-PCR and real-time PCR analyses (Figs. 3B and C). These results collectively suggest that inhibition of ERK1/2 activity by curcumin reduces TP and ERCC1 expression in NSCLC cells.

Knockdown of either TP or ERCC1 expression by siRNA transfection sensitizes NSCLC cells to curcumin. To evaluate the importance of TP and ERCC1 in the curcumin induced cytotoxic effect in NSCLC cells, specific siRNAs against TP and ERCC1 were used to knock down TP and ERCC1 expression in curcumin-treated cells. We found that TP and ERCC1 expression became significantly decreased 24 h after treatment with 200 nM siRNA in curcumin-exposed A549 and H1975 cells (Fig. 4A). Furthermore, curcumin-decreased ERK1/2 phosphorylation was not affected in cells transfected with TP or ERCC1 siRNA. Cell viability was determined by MTS and the colony-forming ability assay, as shown in Figs. 4B and C. The effect of curcumin-induced cytotoxicity was significantly enhanced by transfection with siRNAs against TP and ERCC1 relative to transfection of the curcumin-treated group with the scrambled siRNA ($**P < 0.01$; Figs. 4B and C).

U0126 enhances the curcumin-induced cytotoxicity in NSCLC cells. The MKK1/2 inhibitor U0126 was investigated to determine whether complete inhibition of ERK1/2 activation would promote downregulation of TP and ERCC1 by curcumin. The A549 and H1975 cells were treated with various concentrations of MKK1/2

inhibitor U0126 (2.5–10 µM) and curcumin (5 µM). As shown in Fig. 4D, curcumin

decreased the extent of ERK1/2 phosphorylation, and the TP and ERCC1 protein

levels were significantly further decreased by treatment with U0126 relative to the

control DMSO-treated cells. Next, we evaluated whether ERK1/2 inactivation is

required for curcumin-induced cytotoxicity in NSCLC cells lines. The cells were

cotreated with various concentrations of curcumin and U0126 for 24 h. Cell viability

was assessed by the MTS assay. In Fig. 4E, U0126 enhanced the cytotoxicity in

curcumin-exposed NSCLC cells. These results indicate that inactivation of

MKK1/2–ERK1/2 signal by curcumin suppresses TP and ERCC1 expression and

decreases the viability of the NSCLC cells.

**Curcumin decreases the protein levels of phosphorylated ERK1/2, TP, and
ERCC1 induced by cisplatin.** Next, we examined the combined effects of curcumin

and cisplatin on the expression of TP and ERCC1, and compared with the results of

each separate treatment. As shown in Fig. 5, cisplatin increased the levels of

phosphorylated ERK1/2 in a dose-dependent manner and curcumin suppressed this

activation. No effect on the levels of unphosphorylated ERK1/2 was noted. Moreover,

curcumin was found to decrease the cisplatin-elicited upregulation of TP and ERCC1

protein expression in A549 and H1975 cells (Fig. 5). Furthermore, we examined the

changes in mRNA levels of TP and ERCC1 after treatment with curcumin and

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cisplatin combination by using RT-PCR and real-time PCR analyses. In Figs. 6A and

B, it can be seen that the combination of cisplatin and curcumin produced

significantly lower TP and ERCC1 mRNA levels relative to cisplatin alone. Next, to

evaluate the possible mechanisms for posttranslational and posttranscriptional

regulation of TP and ERCC1 expression, which occur upon cotreatment with

curcumin and cisplatin, the protein and mRNA stability of TP and ERCC1 were

examined. As shown in Figs. 6C and D, curcumin decreased the mRNA and protein

stability of TP and ERCC1 in cisplatin-treated NSCLC cell lines. Taken together,

these results indicate that curcumin downregulates cisplatin-elicited ERK1/2

activation as well as the expression of TP and ERCC1.

Curcumin potentiates the cytotoxicity of cisplatin toward NSCLC cells.

Furthermore, we used dose-inhibition studies to test whether curcumin could sensitize

NSCLC cell lines to cisplatin. The viable cells were evaluated by the MTS assays. In

Fig. 7A, cotreatment with curcumin and cisplatin caused a significant decrease in cell

viability. This indicates increased cisplatin sensitivity (from an IC_{50} of ~20 to 10

µg/mL) with the addition of 20 µM curcumin to A549 cells. Similar phenomena were

observed when cell death was assessed using the trypan blue exclusion assay (Fig.

7B). Additionally, the mean values of the combination index (CI) indicate that the

curcumin–cisplatin treatment has a synergistic effect (Fig. 7C). Moreover, NSCLC

(A549, H520, and H1975) cancer cells were exposed to curcumin and/or cisplatin and their proliferation was determined over 1–4 days after exposure to the drugs. Curcumin alone (20 μM) has an antiproliferative effect on NSCLC. However, curcumin was found to significantly enhance the inhibition of cell growth by cisplatin in NSCLC cells (Fig. 7D). The combination of curcumin and cisplatin together was more effective than either treatment alone.

Influence of ERK1/2 inactivation on the regulation of TP and ERCC1 protein expression and cell viability under curcumin and cisplatin cotreatment. We next tested whether curcumin-dependent ERK1/2 inactivation is essential for synergistic cytotoxicity induced by curcumin and cisplatin. The NSCLC cells were transfected with MKK1/2-CA (constitutively active MKK1/2) vectors, and then treated with curcumin and cisplatin for 24 h. Cell viability was assessed by the MTS assay. Fig. 8A shows that the expression of the MKK1/2-CA vectors rescued the TP and ERCC1 protein levels that were decreased by the curcumin and cisplatin combination. Interestingly, transfection with MKK1/2-CA vectors also enhanced cell survival, which was suppressed by curcumin and/or cisplatin in NSCLC cells (Fig. 8B). Therefore, curcumin enhances the cytotoxicity of cisplatin against NSCLC cells through a mechanism that depends upon inactivation of MKK1/2–ERK1/2.

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Discussion

The results of our study have identified an additional mechanism through which

curcumin induces cytotoxic effects in NSCLC cells via decreasing the expression of

TP and ERCC1. Previous studies indicated that NF-κB (Aggarwal et al., 2006), AKT

(Tomita et al., 2006b), and AP-1 (Tomita et al., 2006a) participate in the mediation of

the antiproliferative effects of curcumin. The number of signaling pathways and

molecular targets involved is continuously growing and consequently the mechanism

is becoming more complex. The mechanism is cell-type specific and dose dependent.

Curcumin inhibits cell proliferation and induces apoptosis in human cancer cells as a

result of the induction of proapoptotic proteins and the inhibition of antiapoptotic

proteins as well as inhibition of survival pathways (Dorai et al., 2001; Radhakrishna

Pillai et al., 2004). For example, curcumin induces apoptosis in human renal cells

through the downregulation of Bcl-xL and IAP, the release of cytochrome c, and the

inhibition of AKT (Woo et al., 2003). The inhibitory effect of curcumin on ERK1/2

activation and the proliferation of pancreatic stellate cells is mediated by inducing the

expression of the heme oxygenase-1 gene (Schwer et al., 2008). We have found that

curcumin inactivates the MKK1/2–ERK1/2 signal in NSCLC cell lines. Enforced

expression of MKK-CA rescues the TP and ERCC1 protein levels that were decreased

by curcumin. In contrast, addition of the MKK1/2 inhibitor U0126 enhances the

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curcumin-elicited downregulation of TP and ERCC1. Knockdown of TP and ERCC1

expression significantly decreases the cell viability in curcumin-exposed NSCLCs. To

our knowledge, the results of this study provide the first indication that curcumin

decreases the expression of TP and ERCC1 by inactivation of MKK1/2–ERK1/2. The

expression of TP and ERCC1 plays a protective role in curcumin-treated NSCLC

cells.

In pancreatic cancer cells, curcumin was shown to augment the cytotoxic effect of

gemcitabine through downregulation of ERK1/2 activation (Lev-Ari et al., 2007).

Curcumin also inhibits the phosphorylation of AKT and enhances the phosphorylation

of p38 MAPK. Additionally, curcumin inhibits the proliferation of cisplatin-resistant

ovarian cancer cells by inducing the generation of superoxide ions, G2/M arrest, and

apoptosis (Weir et al., 2007). Our study also shows that curcumin, as an ERK1/2

inhibitor, decreases cisplatin-induced ERK1/2 activation. Moreover, MKK1/2-CA

transfection rescues the decreased phospho-ERK1/2 levels and improves cell viability,

which was decreased by the cisplatin and curcumin cotreatment. Collectively, these

results lead us to conclude that the antiproliferative effects of the curcumin and

cisplatin combination observed in NSCLC cells are mediated at least in part by

inactivation of ERK1/2.

Curcumin appears to potentiate the cytotoxic effects of the other chemotherapeutic

agents in several different types of cancer cells. For example, curcumin suppresses

paclitaxel-induced expression of the antiapoptotic proteins XIAP, IAP-1, IAP-2, Bcl-2,

and Bcl-xL, and the proliferative proteins cyclooxygenase 2, c-Myc, and cyclin D1

(Aggarwal et al., 2005). Furthermore, curcumin suppresses the paclitaxel-induced

NF-κB pathway in breast cancer cells and inhibits the metastasis of human breast

cancer to the lungs in nude mice (Aggarwal et al., 2005). The combination of

curcumin with paclitaxel augments the anticancer effects of paclitaxel against HeLa

cells (Bava et al., 2005). This synergistic effect is, in part, related to the

downregulation of paclitaxel-induced NF-κB activation and phosphorylation of AKT

pathways by curcumin. In this study, we also found that curcumin could enhance

cisplatin sensitivity in human fibroblasts HFW and embryonic kidney HEK293 cells

(Supplementary Fig. S1). Therefore, curcumin has a large potential in cancer therapy.

Many mechanisms of cisplatin resistance have been proposed, including changes in

cellular uptake and efflux of the drug, inhibition of apoptosis, and increased DNA

repair (Ohmichi et al., 2005). Tumor DNA repair capacity is frequently increased as

an inherent cellular mechanism for evading cell death as a result of administration of

chemotherapeutic drugs. This study and other previous studies show that curcumin

could decrease the expression of DNA repair enzymes that are frequently associated

with chemoresistance (Dhandapani et al., 2007). It has been found that curcumin can

sensitize glioma cells to the action of cisplatin via a mechanism involving downregulation of expression of DNA repair enzymes (MGMT, DNA-PK, Ku70, Ku80, and ERCC-1) (Dhandapani et al., 2007). Moreover, curcumin-mediated inhibition of the Fanconi anemia/BRCA pathway sensitizes tumor cells to cisplatin, resulting in apoptotic death of ovarian and breast tumor cell lines (Chirnomas et al., 2006). Here, we have presented the first evidence that curcumin promotes sensitivity of cisplatin to NSCLC via ERK inactivation–mediated ERCC1 downregulation. Our results support the previous findings that ERCC1 is a promising target for future investigations of treatments or methods to prevent human NSCLC.

Previous studies indicated that protein kinase C (PKC) or Ras has been suggested to be involved in the activation of ERK1/2 by cisplatin (Basu and Tu, 2005; Woessmann et al., 2002). In this study, we found that cisplatin could activate ERK1/2 in human lung cancer cell lines. However, whether PKC or Ras participate in up-regulating ERK1/2 activity in cisplatin-exposed NSCLC cells need further examined. Furthermore, our results indicated that curcumin could decrease cisplatin-elicited the expression of ERCC1 and enhance the sensitivity of cisplatin treatment for NSCLC. It has been reported that Snail (also known as Snail1) transcription factor up-regulates ERCC1 transcription and contributes to cisplatin resistance in head and neck squamous cell carcinoma (HNSCC) cells (Hsu et al.,

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2010). Consistent with our previous study, depletion of endogenous ERCC1 expression by si-ERCC1 RNA transfection significantly enhance cisplatin's cytotoxic effect (Ko et al., 2010). In addition, both increased AP-1 (activator protein 1) levels and increased levels of Jun phosphorylation contribute to the increased transactivation of ERCC1 expression in cisplatin-treated human ovarian carcinoma cells (Li et al., 1998). In present study, we have shown that cisplatin could increase TP protein levels in NSCLC cell lines. Previous findings indicated that TP has cytoprotective functions against cisplatin in human leukemia Jurkat cells which are independent of its enzymatic activity (Ikeda et al., 2003). Moreover, TP confers cytoprotection against DNA damaging agents (cisplatin, UV, VP-16) on tumor cells, and that TP-induced anti-apoptotic effects are at least partly mediated through regulation of the phosphatidyl inositol 3-kinase (PI3K)/AKT survival pathways (Jeung et al., 2006). However, the detail molecular mechanisms that cisplatin regulate TP expression in NSCLC cells is under our investigation.

In conclusion, our study indicates that curcumin synergies with cisplatin via downregulation of TP and ERCC1. This highlights a key aspect of cisplatin resistance in NSCLC. Our results provide valuable insights for the development of future therapies in efforts to overcome cisplatin resistance in the treatment of NSCLC. On the basis of these findings, further investigations of curcumin as a cisplatin sensitizer

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is warranted and animal studies are ongoing to study the effect of combination

curcumin and cisplatin in mouse models of NSCLC.

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Authorship Contributions:

Participated in research design: Lin.

Conducted experiments: Lin, Tsai, Weng, Kuo, and Chiu.

Contributed new reagents or analytic tools: Lin, Tsai, Weng, Kuo, and Chiu.

Performed data analysis: Lin.

Wrote or contributed to the writing of the manuscript: Lin.

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MOLPHARM#071316

Footnotes

MOLPHARM#071316

Figure Legends

Fig. 1. Protein levels of phospho-MKK1/2, phospho-ERK1/2, TP, and ERCC1 were decreased by treatment with curcumin. A, NSCLC cell lines (A549, H520, or H1975) were treated with curcumin (5–40 µM) in complete medium for 24 h. Cells were then extracted for the determination of phospho-MKK1/2, phospho-ERK1/2, TP, ERCC1, ERK1/2, and actin protein levels by western blotting. B and C, after the same treatment described in A, total RNA was isolated and subjected to RT-PCR and real-time PCR for TP and ERCC1. **, $P < 0.01$ and *, $P < 0.05$, using the Student's *t*-test for the comparison between cells treated with or without curcumin in NSCLC cells.

Fig. 2. Treatment with curcumin decreased the cellular TP and ERCC1 mRNA and protein stability in NSCLC cells. A, cells were exposed to curcumin (10 µM) for 9 h in the presence of actinomycin D (2 µg/mL) for 3, 6, or 9 h. After the treatment, total RNA was extracted and analyzed by RT-PCR. TP and ERCC1 mRNA levels were normalized against GAPDH in 3 separate treatments. B, cells were treated with curcumin (10 µM) for 9 h, followed by the addition of cycloheximide (60 µg/mL) for 3, 6, and 9 h. C, cells were treated with curcumin (40 µM) for 18 h, and then with MG132 (10 µM) or lactacystin (10 µM) for 6 h. Whole-cell extracts were collected for western blot analysis of TP and ERCC1 protein levels. D, as described above,

equal amounts of protein in each cell extract was subjected to immunoprecipitation

(IP) with an antiubiquitin antibody. The immunoprecipitates were analyzed by

immunoblot (IB) analysis using an anti-TP or an anti-ERCC1 antibody.

Fig. 3. Transfection of MKK1-CA vectors expression restored the TP and ERCC1

protein levels that were decreased by curcumin. A, MKK1-CA expression vectors

were transfected into A549 and H1975 cells by using Lipofectamine 2000. After

transfection, the cells were treated with curcumin (10–40 μM) for 24 h. Whole-cell

extracts were collected for western blot analysis of TP and ERCC1 protein levels. B

and C, as described above, total RNA was isolated and subjected to RT-PCR and

real-time PCR for TP and ERCC1. a^{**}, $P < 0.01$ using the Student's t-test for

comparison between the cells transfected with MKK1/2-CA or pcDNA3 vectors. b^{**},

$P < 0.01$ (Student's t-test). Comparison between the cells transfected with

MKK1/2-CA or pcDNA3 vectors in curcumin treated cells.

Fig. 4. Knockdown of TP and ERCC1 expression by siRNA transfection enhances

curcumin-induced cell death. A, A549 and H1975 cells were transfected with siRNA

duplexes (200 nM) specific to TP, ERCC1, or a scrambled control in complete

medium for 24 h before treatment with curcumin (5–40 μM) for 24 h. Whole-cell

extracts were collected for western blot analysis using specific antibodies against TP,

ERCC1, phospho-ERK1/2, and actin. B and C, cell viability was determined using the

MTS and colony-forming ability assay. The results (mean ± SEM) were obtained from 3 independent experiments. **, $P < 0.01$, using the Student's t-test for the comparison between cells treated with curcumin in combination with the siRNA against TP RNA, the siRNA against ERCC1 RNA, and the scrambled siRNA. D, A549 and H1975 cells were exposed to curcumin (5 µM) and U0126 (2.5, 5, and 10 µM) for 24 h. Cells were then extracted for western blotting. E, after the treatment described in D, the cell viability was determined using the MTS assay. The results (mean ± SEM) were obtained from 3 independent experiments. **, $P < 0.01$, using the Student's t-test for the comparison between the curcumin-treated cells cotreated with U0126 and the control cells that were not treated with U0126.

Fig. 5. Curcumin decreases cisplatin-induced protein levels of phopho-ERK1/2, TP, and ERCC1. A, A549 or H1975 cells (10^6) were cultured in complete medium for 18 h and then exposed to curcumin (10–40 µM) and cisplatin (10 µg/mL) for 24 h in complete medium. B, cells were exposed to various concentrations of cisplatin and curcumin (20 µM) for 24 h. After treatment, cell extracts were examined by western blots to determine the phospho-ERK1/2, TP, and ERCC1 protein expression levels. **, $P < 0.01$, using the Student's t-test for the comparison between cells treated with cisplatin/curcumin alone and the cells cotreated with these two drugs.

Fig. 6. TP and ERCC1 transcripts are decreased by the combination of curcumin and

cisplatin in NSCLC cells. A and B, A549 or H1975 cells were treated with curcumin

(40 µM) and/or cisplatin (10 µg/mL) for 24 h. Total RNA was isolated and expression

levels of TP and ERCC1 genes were determined by RT-PCR and real-time PCR. **,

$P < 0.01$, using the Student's t-test for the comparison between cells treated with

cisplatin alone and the cells cotreated with curcumin. C, cells were exposed to

cisplatin (10 µg/mL) with or without curcumin (20 µM) for 9 h in the presence or

absence of actinomycin D (2 µg/mL) for 3, 6, or 9 h. After the treatment, total RNA

was isolated and subjected to RT-PCR for TP and ERCC1 transcripts. D, A549, H520,

and H1975 cells were treated with cisplatin (10 µg/mL) alone or co-treated with

curcumin (40 µM) for 9 h, followed by the addition of cycloheximide (60 µg/mL) for

3, 6, and 9 h. Whole-cell extracts were collected for western blot analysis of TP and

ERCC1 protein levels.

Fig. 7. Curcumin enhances the cisplatin-induced cytotoxic effects of NSCLC cell

lines. A, cells were treated with curcumin (5–40 µM) and cisplatin (2–20 µg/mL) for

24 h. The cell viability was determined using the MTS assay. The results (mean ±

SEM) were obtained from 3 independent experiments. B, after the treatment described

above, unattached and attached cells were collected and stained with trypan blue dye.

The stained dead cells were counted manually. The columns indicate the percentage

of trypan blue-positive cells, representing the population of dead cells. The bars

indicate the SEM from 3 independent experiments. The results from the different cell

lines treated with curcumin or cisplatin alone and the combination of these two drugs

were compared. **, $P < 0.01$ (Student's *t*-test). C, the mean of CI values for the

curcumin–cisplatin combination treatment in A549, H520, and H1975 cells. CI values

were averaged for each experiment and the values were used to calculate the mean

between the experiments as described in *Materials and Methods*. The points and

columns indicate the mean values obtained from 3 independent experiments. The bars

indicate the SEM. D, cells were treated with cisplatin (0.5 µg/mL) and/or curcumin

(20 µM) for 1–4 d. The viable cells were determined by the MTS assay on each day in

triplicate. The results (mean ± SEM) were from 4 independent experiments. **, $P <$

0.01, using the Student's *t*-test for the comparison between cells treated with cisplatin

alone or cotreated with curcumin.

Fig. 8. Enforced expression of MKK1/2-CA vectors decreases the curcumin and

cisplatin-induced cytotoxicity. A, cells were transfected with the MKK1/2-CA vectors

for 24 h, and then treated with cisplatin (10 µg/mL) and curcumin (20 µM) for 24 h.

After treatment, cell extracts were examined by western blot for the determination of

phospho-ERK1/2, TP, and ERCC1 protein expression levels. B, cell viability was

determined using the MTS assay. The results (mean ± SEM) were from 4 independent

experiments. The results from different cell lines transfected with MKK1/2-CA or

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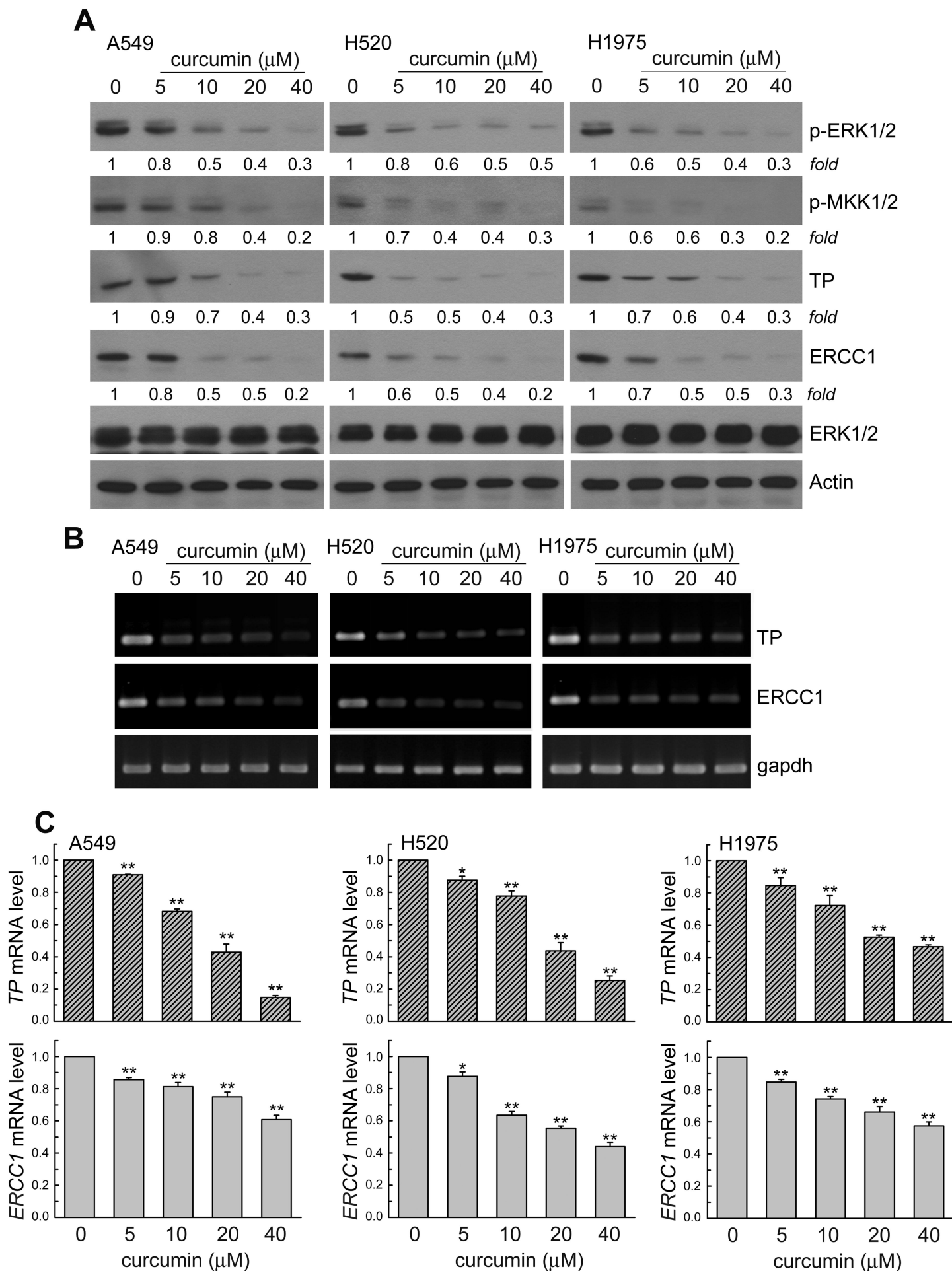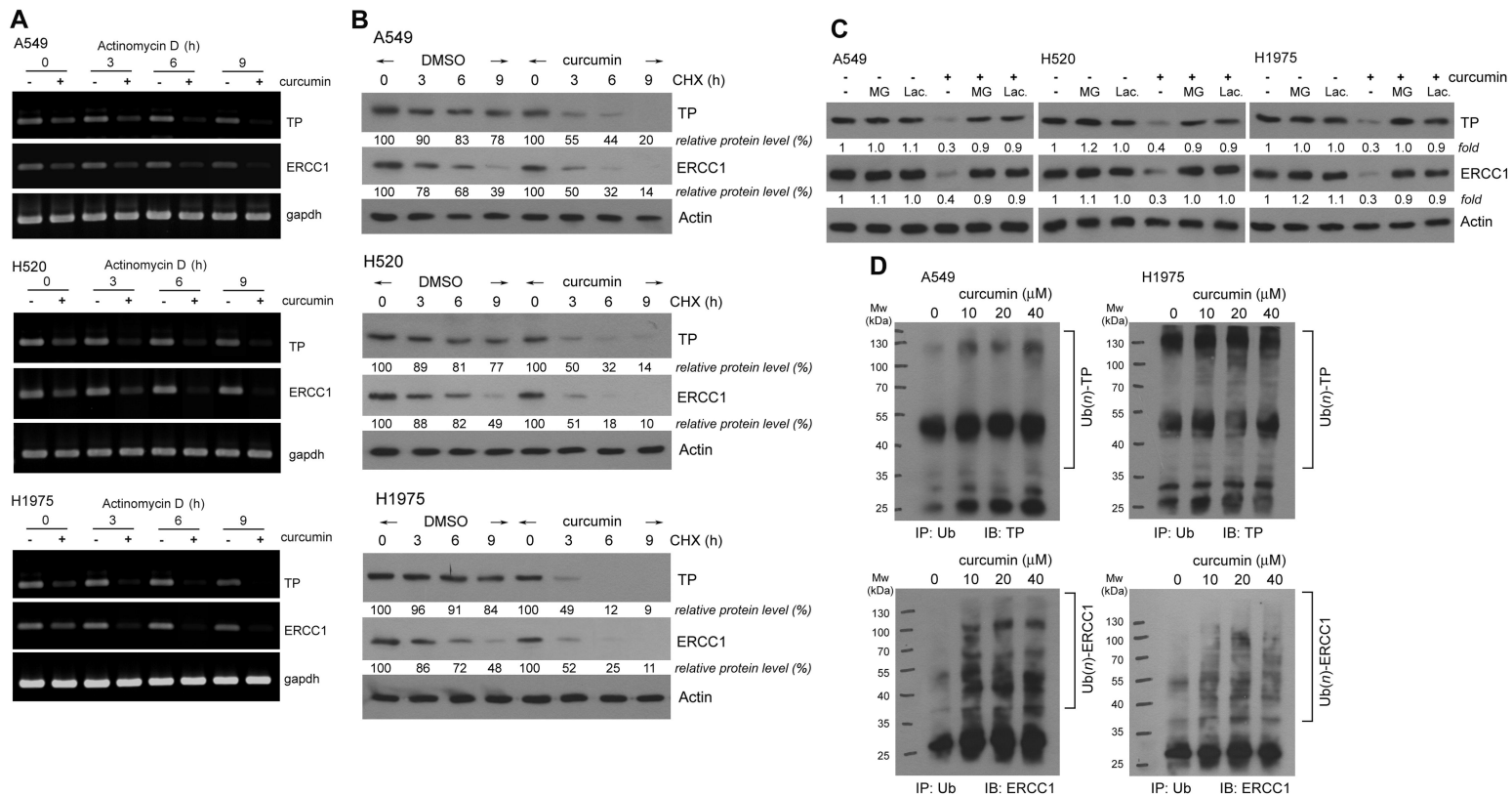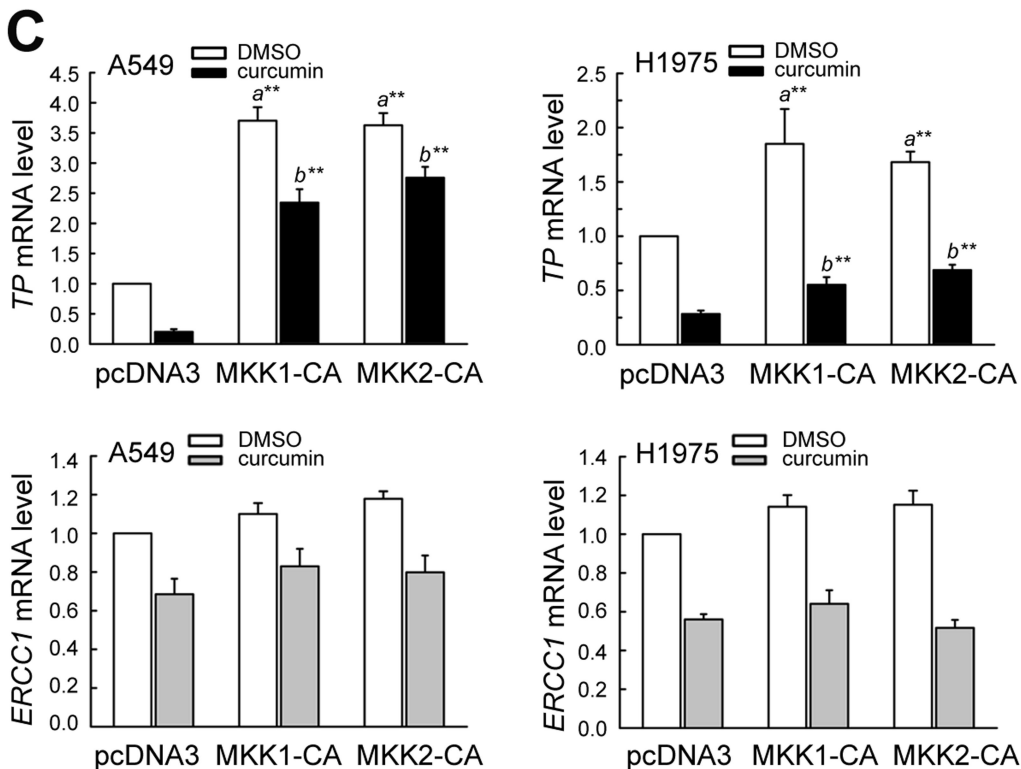pcDNA3 vector were compared. **, $P < 0.01$ and *, $P < 0.05$ (Student's t-test).

Fig. 1

A

A549

Actinomycin D (h)

0		3		6		9	
−	+	−	+	−	+	−	+	curcumin

TP
ERCC1
gapdh

H520

Actinomycin D (h)

0		3		6		9	
−	+	−	+	−	+	−	+	curcumin

TP
ERCC1
gapdh

H1975

Actinomycin D (h)

0		3		6		9	
−	+	−	+	−	+	−	+	curcumin

TP
ERCC1
gapdh

B

A549

← DMSO → ← curcumin →

0	3	6	9	0	3	6	9	CHX (h)

TP
100 90 83 78 100 55 44 20   *relative protein level (%)*
ERCC1
100 78 68 39 100 50 32 14   *relative protein level (%)*
Actin

H520

← DMSO → ← curcumin →

0	3	6	9	0	3	6	9	CHX (h)

TP
100 89 81 77 100 50 32 14   *relative protein level (%)*
ERCC1
100 88 82 49 100 51 18 10   *relative protein level (%)*
Actin

H1975

← DMSO → ← curcumin →

0	3	6	9	0	3	6	9	CHX (h)

TP
100 96 91 84 100 49 12 9   *relative protein level (%)*
ERCC1
100 86 72 48 100 52 25 11   *relative protein level (%)*
Actin

C

A549

−	−	−	+	+	+	curcumin
−	MG	Lac.	−	MG	Lac.	

TP
1 1.0 1.1 0.3 0.9 0.9   *fold*
ERCC1
1 1.1 1.0 0.4 0.9 0.9   *fold*
Actin

H520

−	−	−	+	+	+	curcumin
−	MG	Lac.	−	MG	Lac.	

TP
1 1.2 1.0 0.4 0.9 0.9   *fold*
ERCC1
1 1.1 1.0 0.3 1.0 1.0   *fold*
Actin

H1975

−	−	−	+	+	+	curcumin
−	MG	Lac.	−	MG	Lac.	

TP
1 1.0 1.0 0.3 1.0 0.9   *fold*
ERCC1
1 1.2 1.1 0.3 0.9 0.9   *fold*
Actin

D

A549

Mw (kDa)   curcumin (μM)
0 10 20 40

130
100
70
55
40
35
25

Ub(n)-TP

IP: Ub   IB: TP

H1975

Mw (kDa)   curcumin (μM)
0 10 20 40

130

55

35
25

Ub(n)-TP

IP: Ub   IB: TP

Mw (kDa)   curcumin (μM)
0 10 20 40

130
100
55
40
35
25

Ub(n)-ERCC1

IP: Ub   IB: ERCC1

Mw (kDa)   curcumin (μM)
0 10 20 40

130
55
40
35
25

Ub(n)-ERCC1

IP: Ub   IB: ERCC1

Fig. 2

A

A549

←	pcDNA3		→	←	MKK1-CA		→	
0	10	20	40	0	10	20	40	curcumin (μM)

p-ERK1/2

| 1 | 0.6 | 0.4 | 0.3 | 2.4 | 1.4 | 1.4 | 1.3 | *fold* |

TP

| 1 | 0.6 | 0.4 | 0.3 | 1.8 | 1.3 | 1.3 | 1.2 | *fold* |

ERCC1

| 1 | 0.5 | 0.5 | 0.3 | 2.1 | 2.0 | 2.0 | 1.9 | *fold* |

HA-MKK1-CA

ERK1/2

H1975

←	pcDNA3		→	←	MKK1-CA		→	
0	10	20	40	0	10	20	40	curcumin (μM)

p-ERK1/2

| 1 | 0.4 | 0.3 | 0.3 | 2.0 | 1.6 | 1.5 | 1.5 | *fold* |

TP

| 1 | 0.5 | 0.4 | 0.3 | 2.1 | 1.1 | 0.9 | 0.9 | *fold* |

ERCC1

| 1 | 0.5 | 0.4 | 0.3 | 2.2 | 2.0 | 2.0 | 1.8 | *fold* |

HA-MKK1-CA

ERK1/2

B

A549

pcDNA3		MKK1-CA		MKK2-CA		
-	+	-	+	-	+	curcumin

TP

ERCC1

gapdh

H1975

pcDNA3		MKK1-CA		MKK2-CA		
-	+	-	+	-	+	curcumin

TP

ERCC1

gapdh

C

Fig. 3

Fig. 4

A

A549

								cisplatin (10 µg/mL)
-	-	-	-	+	+	+	+	
0	10	20	40	0	10	20	40	curcumin (µM)

p-ERK1/2
1   0.6   0.5   0.3   2.2   0.7   0.7   0.6   *fold*

TP
1   0.4   0.4   0.3   2.4   0.7   0.7   0.7   *fold*

ERCC1
1   0.5   0.4   0.4   2.1   0.6   0.6   0.5   *fold*

Actin

ERK1/2

H1975

p-ERK1/2
1   0.7   0.4   0.4   2.4   0.8   0.7   0.5   *fold*

TP
1   0.5   0.4   0.3   2.3   0.6   0.5   0.5   *fold*

ERCC1
1   0.5   0.4   0.3   2.2   0.5   0.5   0.5   *fold*

Actin

ERK1/2

B

A549

								curcumin (20 µM)
-	-	-	-	+	+	+	+	
0	2	5	10	0	2	5	10	cisplatin (µg/mL)

p-ERK1/2
1   2.0   2.0   2.2   0.5   0.6   0.7   0.7   *fold*

TP
1   2.2   2.3   2.4   0.4   0.5   0.6   0.8   *fold*

ERCC1
1   2.0   2.0   2.1   0.3   0.5   0.6   0.6   *fold*

Actin

ERK1/2

H1975

p-ERK1/2
1   2.0   2.3   2.4   0.4   0.7   0.7   0.7   *fold*

TP
1   2.3   2.3   2.4   0.4   0.5   0.5   0.6   *fold*

ERCC1
1   1.9   2.1   2.2   0.4   0.4   0.4   0.4   *fold*
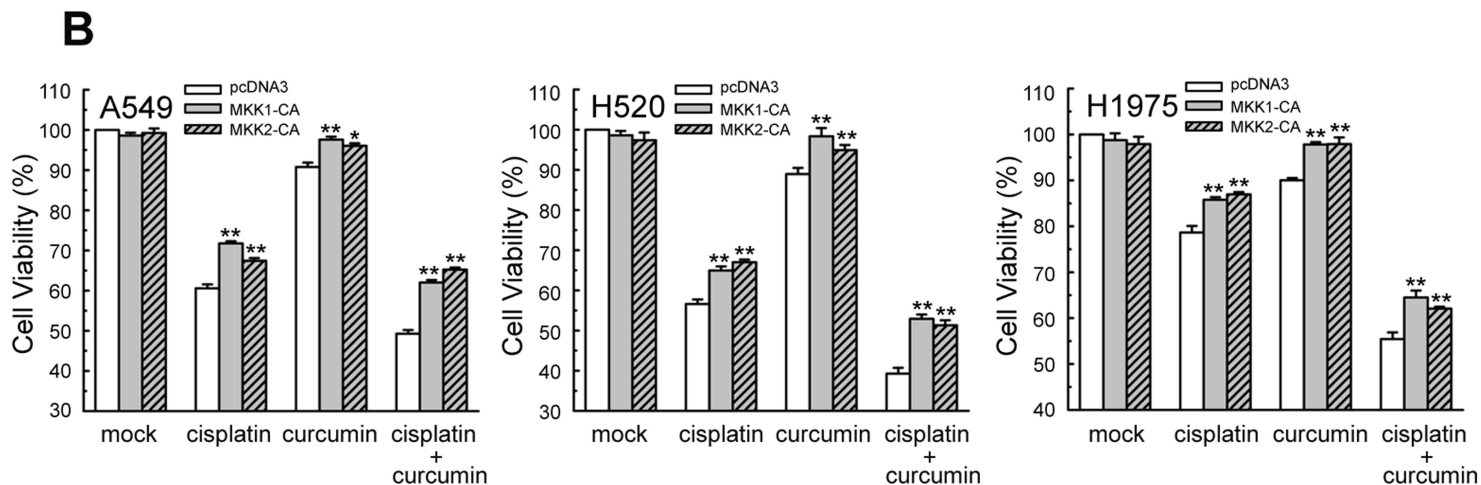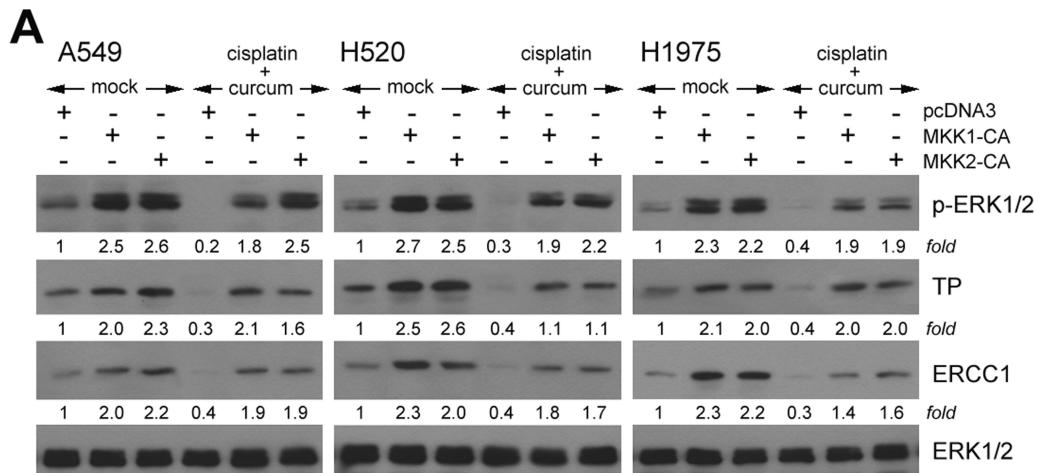
Actin

ERK1/2

Fig. 5

Fig. 6

Fig. 7

Fig. 8

Synergistic effect of curcumin and cisplatin via downregulation of thymidine phosphorylase and ERCC1

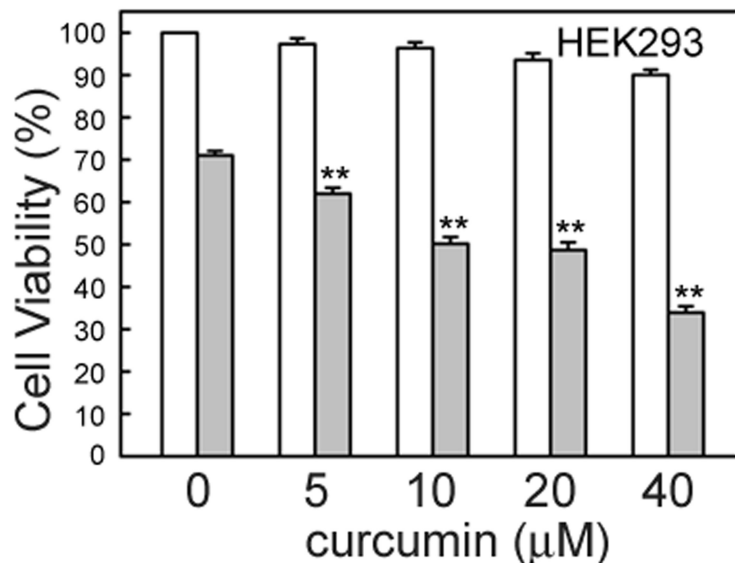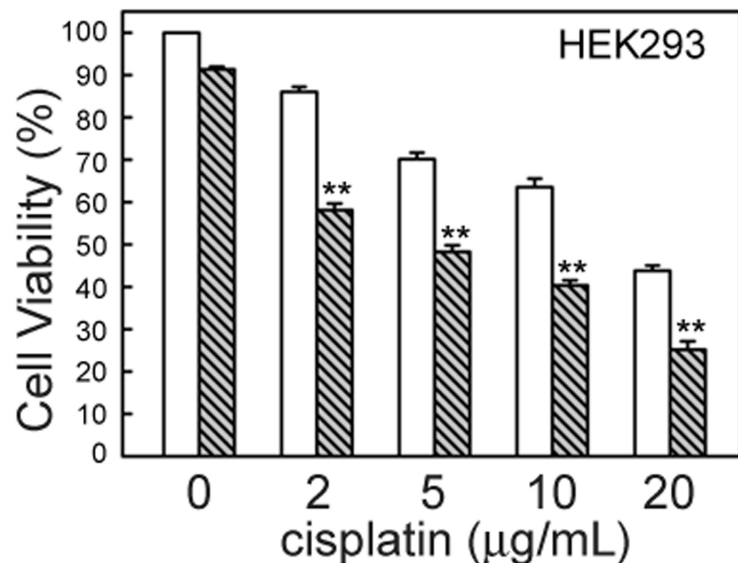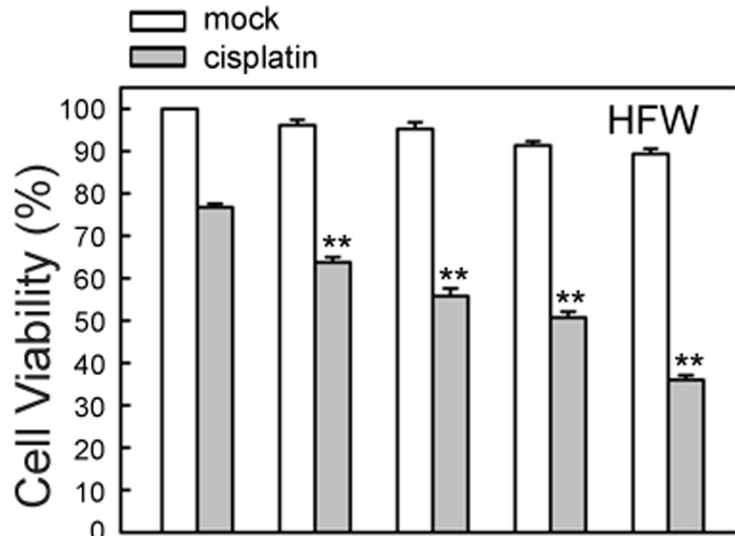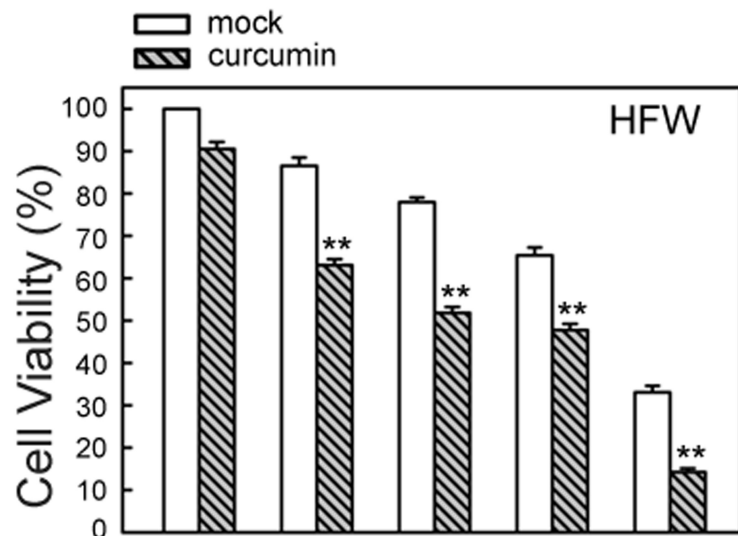**Min-Shao Tsai, Shao-Hsing Weng, Ya-Hsun Kuo, Yu-Fan Chiu, and Yun-Wei Lin***

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Legends for Supplementary Figures

Supplementary Fig. S1. Curcumin enhances the cisplatin-induced cytotoxic effects of human fibroblasts HFW and human embryonic kidney HEK293 cells. Left panel, cisplatin (2–20 μg/mL) and curcumin (20 μM) was co-added to HFW or HEK293 cells for 24 h. Right panel, cells were treated with different concentration of curcumin and cisplatin (5 μg/mL) for 24 h. The cell viability was determined using the MTS assay. The results (mean ± SEM) were from 3 independent experiments. **, $P < 0.01$, using the Student's t-test for the comparison between cells treated with cisplatin or curcumin alone or with curcumin–cisplatin cotreatment.

Supplemental Figure S1