Luteolin sensitizes the anticancer effect of cisplatin via c-Jun NH_2-terminal kinase–mediated p53 phosphorylation and stabilization

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Abstract

Luteolin is an important flavonoid with a potential anticancer effect. In this study, we examined the molecular mechanisms involved in the sensitization effect of luteolin on cancer cell killing induced by cisplatin, an important cancer chemotherapeutic agent. First, we provided evidence that the sensitization effect of luteolin on cisplatin-induced apoptosis is p53 dependent, as such effect is only found in p53 wild-type cancer cells but not in p53 mutant cancer cells. Moreover, knockdown of p53 by small interfering RNA made p53 wild-type cancer cells resistant to luteolin and cisplatin. Second, we observed a significant increase of p53 protein level in luteolin-treated cancer cells without increase of p53 mRNA level, indicating the possible effect of luteolin on p53 posttranscriptional regulation. Third, we identified the critical role of c-Jun NH_2-terminal kinase (JNK) in regulation of p53 protein stability: luteolin activates JNK, and JNK then stabilizes p53 via phosphorylation, leading to reduced ubiquitination and proteasomal degradation. Finally, by using an *in vivo* nude mice xenograft model, we confirmed that luteolin enhanced the cancer therapeutic activity of cisplatin via p53 stabilization and accumulation. In summary, data from this study reveal a novel molecular mechanism involved in the anticancer effect of luteolin and support its potential clinical application as a chemosensitizer in cancer therapy. [Mol Cancer Ther 2007;6(4):1338–47]

Introduction

Cisplatin (*cis*-diamminediachloroplatium) and its derivatives are among one of the most effective anticancer drugs used clinically in the treatment of solid tumors, including ovarian, testicular, cervical, and small cell lung cancers (1). The anticancer effect of cisplatin mainly depends on its DNA-damaging activity, via its direct interaction with DNA to form DNA adducts (2). Subsequently, several signaling transduction pathways are activated, including ataxia telangiectasia mutated kinase, ataxia telangiectasia and rad3-related kinase, p53, and mitogen-activated protein kinases, leading to apoptotic cell death (3, 4). Among them, p53 activation is one of the major factors responsible for the apoptotic cell death induced by cisplatin (1, 5). It has been observed that cisplatin can cause apoptosis in wild-type p53 cancer cells but not in p53-deficient or mutant cancer cells, suggesting that p53 is the key regulator for cisplatin-mediated apoptosis in cancer cells (6, 7).

p53 is one of the most important tumor suppressor genes in human cancer (8, 9). One important issue in understanding the anticancer function of p53 is the regulation of p53 protein stability (10). Although the exact mechanism of such regulation remains to be fully understood, it is known that its interaction with mouse double minute 2 (MDM2), as well as a number of other proteins, plays a critical role in keeping p53 protein at a low level (10, 11). Another important mechanism regulating p53 protein stability is related to its phosphorylation status (10, 12). For example, DNA damage–activated ataxia telangiectasia mutated kinase and ataxia telangiectasia and rad3-related kinase can phosphorylate p53 on Ser^{15} and Ser^{37} (13), and checkpoint kinase 2 is among the kinases that contribute to the phosphorylation of p53 on Ser^{20} (14). Such phosphorylation then stabilizes the p53 protein level via MDM2-dependent or MDM2-independent pathways (10, 15).

Luteolin, an important flavonoid present in a variety of edible plants, exhibits a wide spectrum of pharmacologic properties, including anticancer properties (16–19). It has been well studied that luteolin is capable of inducing cell cycle arrest or apoptosis in various human cancer cells (20–24). We have reported previously that luteolin is capable of sensitizing apoptotic cell death induced by tumor necrosis factor or tumor necrosis factor–related apoptosis-inducing ligand in various human cancer cells (25, 26), suggesting the potential therapeutic value of luteolin in cancer therapy.

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Note: R. Shi and Q. Huang contributed equally to this work.

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The resistance by tumor cells to cisplatin is one of the major limitations in cisplatin chemotherapy (3, 27). In this study, we aimed to evaluate the effect of luteolin on the chemotherapeutic efficacy of cisplatin, using both *in vitro* cell culture and an *in vivo* cancer xenograft model. We reported here that luteolin significantly enhanced the anticancer effects of cisplatin by sensitizing cisplatin-induced apoptosis. The molecular mechanism responsible for this enhancement activity is found to be closely related to p53: luteolin is capable of stabilizing p53 via enhanced protein phosphorylation and decreased ubiquitination, a process related to c-Jun NH_2-terminal kinase (JNK) activation. Results from this study thus provide new evidence for the potential application of luteolin as a chemosensitizer in cancer therapy.

Materials and Methods

Reagents and Chemicals

Luteolin, 4′,6-diamidino-2-phenylindole (DAPI), cisplatin, and anti-Bax 6A7 antibody were all purchased from Sigma (St. Louis, MO). Pan-caspase inhibitor z-VAD-fmk, JNK inhibitor SP600125, and anti-MDM2 antibody were from Calbiochem (San Diego, CA). Antibodies against caspase-3, phosphorylated p53 (Ser^{15}), phosphorylated p53 (Thr^{81}), phosphorylated JNK (Thr^{183}/Tyr^{185}), JNK, phosphorylated p38 (Thr^{180}/Tyr^{182}), p38, and poly(ADP-ribose) polymerase antibodies were from Cell Signaling Technology (Beverly, CA). Antibodies against ubiquitin, Bax, tubulin, and p53 for Western blot were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody against p53 for immunohistochemical staining is purchased from DAKO (Glostrup, Denmark). All other common chemicals were from Sigma.

Cell Culture and Treatments

Human liver cancer cells HepG2 and Hep3B and human colorectal cancer cells HT29 and HCT116 were from the American Type Culture Collection (Manassas, VA), and human nasopharyngeal cancer cell CNE1 was obtained from Sun Yet-sat University (Guangzhou, China). HepG2, Hep3B, HCT116, and CNE1 were maintained in DMEM (Sigma) with 10% fetal bovine serum (Hyclone, Logan, UT). HT29 cells were maintained in McCoy's 5A medium with 10% fetal bovine serum.

Apoptosis Assessment: DAPI Staining

The cells undergoing apoptosis were evaluated by chromatin condensation, nuclear shrinkage, and formation of apoptotic bodies, all visualized with DAPI staining (28). After various designated treatments, medium was removed, and cells were fixed with 70% ethanol at room temperature for 10 min. Cells were then stained with 0.3 μg/mL DAPI (in PBS) at room temperature for 10 min and visualized under an inverted fluorescence microscope and photographed.

Transient Transfection and RNA Interference

Dominant-negative forms of HA-JNKK1 and HA-JNKK2 were kindly provided by Dr. Zheng-Gang Liu (National Cancer Institute, NIH). Empty vector (pcDNA) and red-fluorescent protein expression vector (pDsRed) were from Clontech (Palo Alto, CA). Transient transfection was done in MDA-MB-231 cells using LipofectAMINE 2000 according to the manufacturer's protocol. All transfections were normalized for total DNA using vector plasmid. For the RNA interference study, FITC-labeled synthetic small interfering RNA (scrambled siRNA and p53 siRNA) were from QIAGEN (Valencia, CA). HCT116 cells were transfected with using the LipofectAMINE 2000 transfection reagent (Invitrogen, Carlsbad, CA). The knockdown efficiency was confirmed by Western blotting.

Immunoprecipitation, Cell Fractionation, and Western Blot

Cells were lysed in CHAPS lysis buffer [150 mmol/L NaCl, 10 mmol/L HEPES (pH 7.4), and 1% CHAPS] for 1 h on ice. The supernatant was collected after centrifugation at 20,000 × *g* for 15 min. Each sample was added with 0.5 μg anti-Bax 6A7 antibody or anti-p53 antibody and 50 μL protein A/G agarose beads (Roche, Indianapolis, IN) and rotated overnight at 4°C. The beads were washed four times using ice-cold PBS buffer and then eluted using SDS sample buffer before subject to Western blot analysis (29). For cell fractionation, treated cells were suspended in 100 μL of buffer [20 mmol/L HEPES, 10 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, 0.1 mmol/L phenylmethylsulfonyl fluoride, 250 mmol/L sucrose (pH 7.5)] and homogenized by a syringe with a 27-gauge needle for 15 to 20 times. The lysate was centrifuged at 1,000 × *g* for 10 min to spin down the intact cells and nuclear. The supernatant was further centrifuged at 15,000 × *g* for 1 h to fractionate mitochondrial and cytosolic fraction (26). For Western blot, equal amount of proteins were fractionated on SDS-polyacrylamide gel in the Mini-PROTEAN II system (Bio-Rad, Hercules, CA) and blotted onto polyvinylidene difluoride membrane (Millipore, Bedford, MA). After blocking with 5% nonfat milk in TBST [10 mmol/L Tris-HCl (pH 7.5), 100 mmol/L NaCl, and 0.1% Tween 20], the membrane was probed with various antibodies and developed with enhanced chemiluminescence (Pierce, Rockford, IL) using a Kodak Image Station (Kodak, Rochester, NY). Densitometric measurements of the bands in Western blot analysis were done using digitalized scientific software program Kodak 1D 3.5 (Kodak).

In vivo Xenograft Experiment

To assess the effect of luteolin on the anticancer efficacy of cisplatin under *in vivo* condition, we used a nude mice xenograft model. The protocol was approved by the University Institutional Animal Care and Use Committee. Briefly, female BALB/c nude mice of 5 to 6 weeks old were purchased from the Animal Resources Centre (Murdoch, Australia) and maintained in specific pathogen-free facility. The mice were inoculated s.c. in the two sides of flanks with 1×10^7 HCT116 cells in a volume of 100 μL of PBS. One week after inoculation, mice bearing with visible tumors were randomly assigned to four experimental groups (six mice per group): control (PBS), luteolin (40 mg/kg body weight), cisplatin (1.25 mg/kg body

weight), and luteolin (40 mg/kg body weight) plus cisplatin (1.25 mg/kg body weight). The dose of cisplatin we used was much lower than that in other studies ranging from 3 to 6 mg/kg (30–32), and cisplatin alone at this dose is not toxic to the mice based on the preliminary experiments. The dose of luteolin was also based on our preliminary study and was comparable with that of other flavonoids used *in vivo* (31). Cisplatin and luteolin were administered together through i.p. injection thrice per week (every Monday, Wednesday, and Friday) with close monitoring of the general conditions of the animals. After 3 weeks, all mice were sacrificed by CO_2 inhalation. The tumors were isolated, and tumor weight and size were measured. The excised tumor tissue was then fixed for immunohistochemical analysis.

Immunohistochemical Staining for p53

The p53 protein level in xenografted tumor tissues was evaluated by immunohistochemical staining. The tumor tissues obtained above were fixed in buffered formalin (10%) for paraffin sectioning. The p53 protein level was detected by immunohistochemistry with light counterstaining using hematoxylin.

Results

Luteolin Expedites and Enhances Cisplatin-Induced Apoptosis in Human Cancer Cells

It is known that cisplatin-induced apoptotic cell death is a relatively slow process, normally requiring 2 to 3 days (33, 34). In this study, among the three cancer cells tested, no cell death was observed in HepG2 and HCT116 cells when treated with 10 μg/mL cisplatin alone for 24 h (Fig. 1A). Evident apoptosis occurred only after 48 h in these two cell lines (data not shown). On the other hand, cisplatin alone (10 μg/mL × 24 h) caused about 20% apoptotic cells in CNE1 cells (Fig. 1A). Luteolin treatment alone (40 μmol/L × 24 h) did not induce evident apoptosis in any of these cells. Interestingly, when cells were pretreated with luteolin for 2 h, cisplatin was able to induced significant cell death in all three cancer cells in a dose-dependent manner (Fig. 1A), suggesting that luteolin is able to expedite as well as to enhance cisplatin-induced apoptotic cell death in those cancer cells. Figure 1B shows the typical chromatin condensation in apoptotic cells induced by combined treatment of luteolin and cisplatin in HCT116 cells, using DAPI staining.

The apoptosis induced by luteolin and cisplatin was further examined by detection of poly(ADP-ribose) polymerase cleavage and caspase activation, the two hallmarks of apoptosis. In HCT116 cells, either luteolin or cisplatin alone caused no caspase-3 or poly(ADP-ribose) polymerase cleavage (Fig. 1C). However, their combination led to evident cleavage of caspase-3 and poly(ADP-ribose) polymerase (Fig. 1C), which is consistent with the cell death evaluated by DAPI staining (Fig. 1A). Furthermore, the cleavage of poly(ADP-ribose) polymerase and caspase-3 were inhibited by the pan-caspase inhibitor z-VAD-fmk (Fig. 1C), suggesting that the apoptosis induced by luteolin and cisplatin is caspase dependent.

p53 Is Required for Enhanced Apoptosis by Luteolin

To elucidate the possible mechanism involved in the sensitization effect of luteolin on cisplatin-induced apoptosis, we first examined the changes of several important

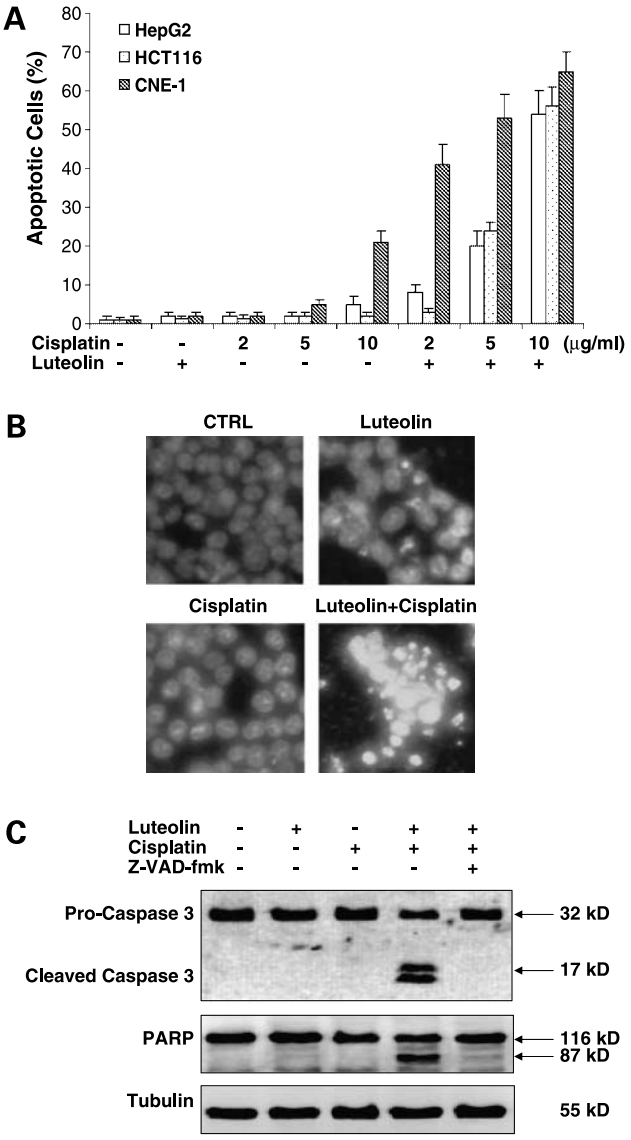

Figure 1. Luteolin enhances cisplatin-induced apoptosis in cancer cells. **A,** cells were pretreated with luteolin for 2 h (40 μmol/L for HCT116 and CNE-1 and 20 μmol/L for HepG2) and then treated with indicated concentrations of cisplatin (μg/mL) for another 24 h. Cells were fixed with 70% ethanol for 5 min and then stained with 0.3 μmol/L DAPI for 10 min. Apoptotic cells were counted according to their morphologic changes under a fluorescence microscope. *Columns,* mean from three independent experiments; *bars,* SD. **B,** HCT116 cells were treated with cisplatin (10 μg/mL) for 24 h with or without luteolin (40 μmol/L × 2 h) pretreatment. Cells were fixed with 70% ethanol for 5 min and then stained with 0.3 μmol/L DAPI for 10 min. Pictures were taken under an inverted fluorescence microscope. **C,** HCT116 cells were treated with luteolin (40 μmol/L × 2 h) followed by cisplatin (10 μg/mL) for 24 h, with or without the presence of z-VAD-fmk (25 μmol/L, 30-min pretreatment). Cells were then collected for detection of PARP and caspase-3 using Western blot. Tubulin was used to serve as a loading control.

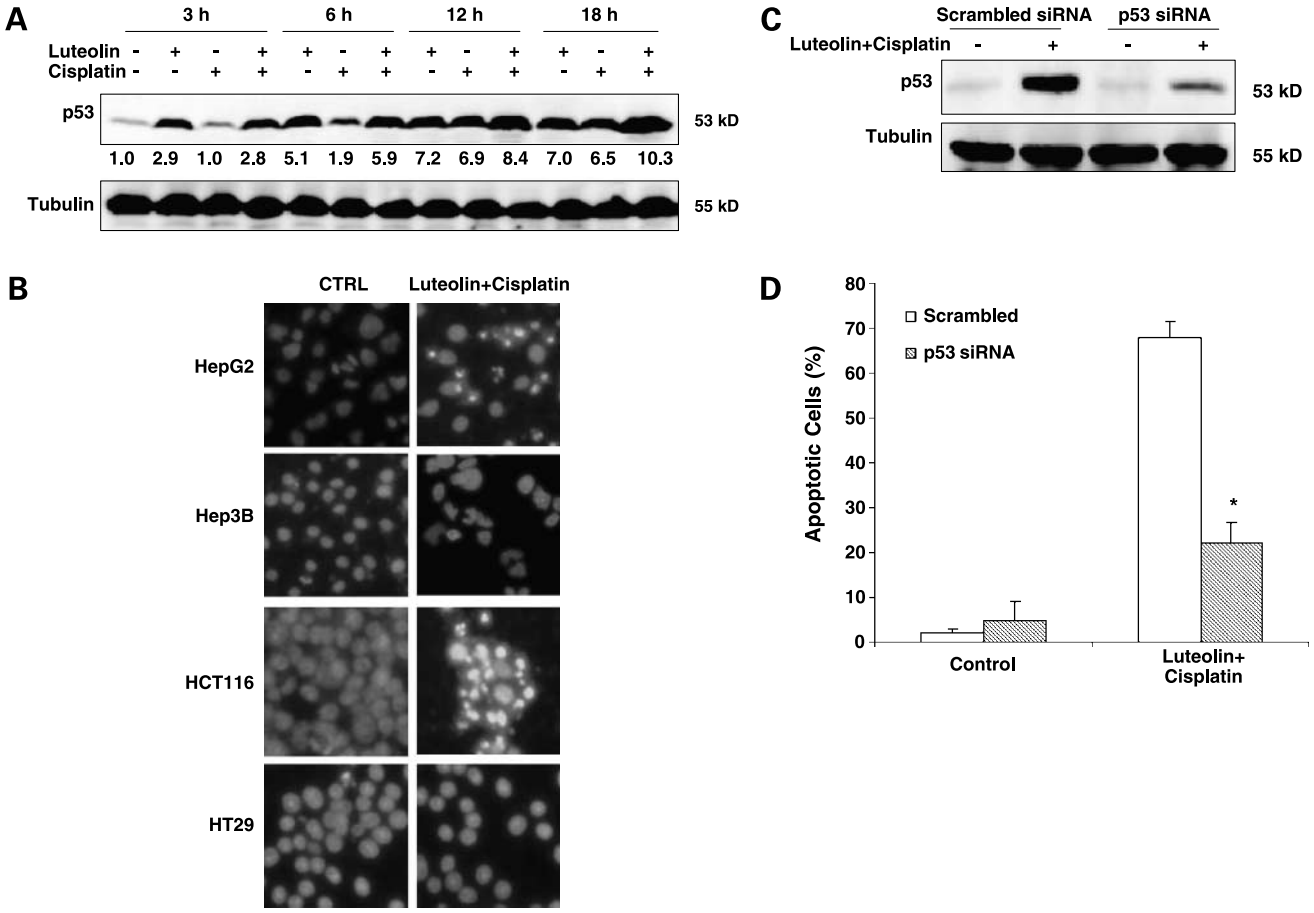

Figure 2. p53 is required for apoptosis induced by luteolin and cisplatin in cancer cells. **A,** HCT116 cells were treated with cisplatin (10 μg/mL) with or without luteolin pretreatment (40 μmol/L × 2 h). After indicated periods of luteolin treatment, cells were collected for detection of p53 protein levels by Western blot. **B,** cells were pretreated with luteolin for 2 h (40 μmol/L for HCT116 and HT29 and 20 μmol/L for HepG2 and Hep3B) and then treated with cisplatin (10 μg/mL) for another 24 h. Cell death was evaluated as described in Fig. 1B. **C,** HCT116 cells were transfected with scrambled siRNA labeled with FITC, or p53 siRNA for 24 h. Then cells were treated with luteolin (40 μmol/L × 2 h) followed by cisplatin (10 μg/mL × 6 h) and collected for detection of p53 by Western blot. **D,** HCT116 cells were transfected with scrambled siRNA or p53 siRNA for 24 h and were treated with luteolin (40 μmol/L × 2 h) followed by cisplatin (10 μg/mL × 24 h). Cell death was evaluated using DAPI staining. *Columns,* mean from three independent experiments; *bars,* SD. *, $P < 0.05$, compared with the group transfected with scrambled siRNA and treated with luteolin and cisplatin.

apoptosis regulatory proteins, such as X-linked inhibitor of apoptosis, Bcl-2, Bcl-X_L, and Bax, in cells treated with luteolin and cisplatin. However, none of them showed any significant changes in HCT116 cells (data not shown). Because p53 is known to be the key regulator for cisplatin-mediated apoptosis in cancer cells (3, 5), we next examined the change of p53 protein in HCT116 cells treated with luteolin, cisplatin, or their combination. Significant increase of p53 protein level was observed as early as 3 h after luteolin treatment, much faster than cisplatin-induced p53 increase that only occurred after treated with cisplatin for 6 h (Fig. 2A). More importantly, combined treatment of luteolin and cisplatin further increased the p53 protein level, especially at 12 and 18 h after treatment, suggesting that p53 is likely to be important in promoting cisplatin-induced apoptosis.

To further test the involvement of p53 in apoptosis induced by combined treatment of luteolin and cisplatin, we compared the responses of cells with different genetic features of p53. HepG2 and HCT116 cells contain wild-type p53 proteins, whereas Hep3B and HT29 cells are with mutant p53 (35–37). As shown in Fig. 2B, luteolin plus cisplatin induced significant apoptosis in HepG2 and HCT116 cells, but not in Hep3B and HT29 cells. Therefore, it is believed that induction of apoptosis by luteolin and cisplatin is dependent on the presence of functional p53. This result is in agreement with previous reports that cisplatin induces apoptosis in a p53-dependent manner (12).

To further confirm the role of p53 in apoptosis induced by luteolin and cisplatin, we knocked down p53 protein in HCT116 cells using the technique of RNA interference. The transfection efficiency of siRNA was higher than 90%, as monitored by a FITC-labeled siRNA (data not shown). The knockdown efficiency was confirmed by Western blot 1(Fig. 2C). Luteolin plus cisplatin elevated p53 protein level in cells transfected with scrambled siRNA, whereas

the p53 protein level was significantly reduced in cells transfected with p53 siRNA. Consistently, p53 knockdown significantly reduced the number of apoptotic cells induced by luteolin and cisplatin (Fig. 2D).

Luteolin Elevates p53 by Increasing Its Protein Stability

One important finding from Fig. 2A is that luteolin alone can elevate p53 protein level significantly and rapidly. In the presence of luteolin, a significant increase of p53 protein was found as early as 3 h, which is much faster than the changes induced by cisplatin. Such a kinetic difference suggests that luteolin and cisplatin promotes p53 protein accumulation via different mechanisms. Because luteolin did not affect p53 mRNA level up to 12 h (data not shown), it is thus possible that the rapid elevation of p53 protein level by luteolin is regulated at posttranscriptional level. To support this hypothesis, we next examined the p53 stability in cells treated with luteolin. In the presence of cycloheximide, a *de novo* protein synthesis inhibitor, the p53 protein was rapidly degraded, whereas luteolin can significantly stabilize p53 protein and prolong its half-life (Fig. 3A).

Luteolin Enhances Phosphorylation of p53 at Thr^{81} and Ser^{15}

The stability of p53 protein is mainly regulated by its phosphorylation and interaction with specific ubiquitin ligases, such as MDM2, that promote its ubiquitination and proteasomal degradation (11, 38). We therefore examined whether luteolin leads to the increased levels of p53 phosphorylation using antibodies against phosphorylated p53 at different phosphorylation sites. As shown in Fig. 3B, shortly after luteolin treatment (30 min), there was a dramatic increase in phosphorylated p53 levels at Thr^{81} and Ser^{15}. Notably, the increase of p53 protein phosphorylation precedes the changes of total p53 protein level as shown in Fig. 2A. At the same time, luteolin treatment did not alter the protein level of MDM2 (Fig. 3B). These findings thus suggest that luteolin might stabilize p53 protein by affecting its protein phosphorylation status.

Critical Role of JNK in Luteolin-Mediated p53 Stabilization and Apoptosis

There are a number of protein kinases that are able to phosphorylate p53 at different sites, among which the mitogen-activated protein kinases, including JNKs, p38, and extracellular signal-regulated kinases, have been well studied (39). Ser^{15} in p53 is the target of both activated JNKs and p38, and Thr^{81} is the target of activated JNKs (39). Here, we thus examined the effect of luteolin on JNK and p38 activation. As shown in Fig. 4A, luteolin treatment caused a significant and sustained JNK activation, without affecting p38 activation (Fig. 4B). Meanwhile, we also tested the effect of cisplatin on JNK activation and observed minimal changes (data not shown). Furthermore, we assessed the effect of the synthetic JNK inhibitor SP600125 on JNK activation and p53 phosphorylation induced by luteolin. As expected, SP600125 pretreatment prevented JNK activation caused by luteolin (Fig. 4C). More importantly, it also blocked luteolin-induced p53 phosphorylation at Thr^{81} and Ser^{15} (Fig. 4C). Taken together, these results clearly show that luteolin may stabilize p53 via JNK activation and subsequent phosphorylation of p53 at Thr^{81} and Ser^{15}.

To further confirm the role of JNK in luteolin- and cisplatin-induced cell death, we next tested the effect of the JNK inhibitor SP600125 on cell death in HCT116 cells treated with luteolin and cisplatin. As shown in Fig. 4D, with SP600125 pretreatment, the number of apoptotic cells induced by luteolin and cisplatin decreased significantly from 58.2% to 7.8%. Similar results were also observed when JNK activation was blocked using a genetic approach. HCT116 cells were transiently transfected with dominant-negative forms of JNKK1 and JNKK2, together with a red-fluorescent protein expression vector (pDsRed) as a transfection marker. As shown in Fig. 4E, expression of dominant-negative (DN) forms of JNKK1 and JNKK2 was able to block the JNK phosphorylation induced by luteolin. Furthermore, cells with successful transfection of HA-JNKK1(DN) and HA-JNKK2(DN) were largely resistant to apoptosis induced by luteolin and cisplatin.

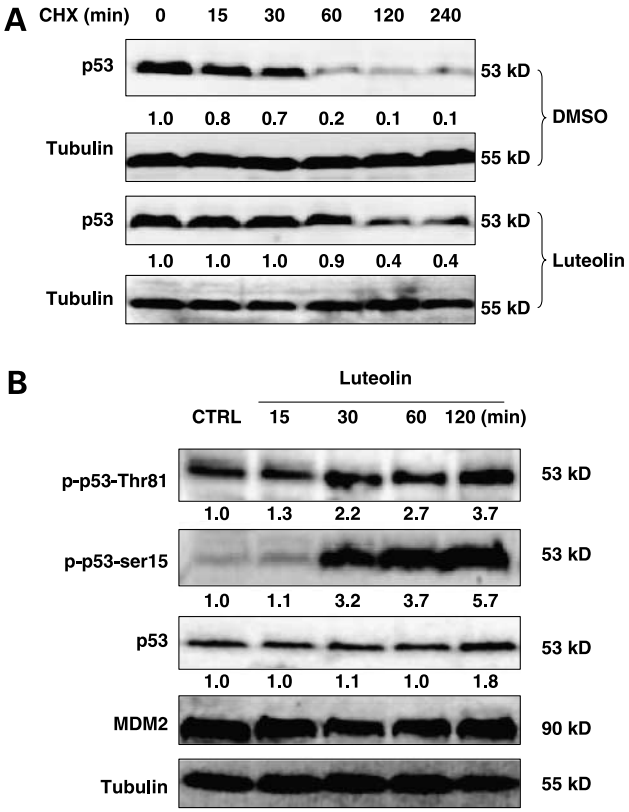

Figure 3. Luteolin enhances p53 protein level by increasing p53 phosphorylation. **A,** HCT116 cells were treated with luteolin (40 μmol/L) or DMSO for 30 min followed by cycloheximide (*CHX*; 1 μg/mL) for the indicated period. Cells were collected for detection of p53 using Western blot. **B,** HCT116 cells were treated with luteolin (40 μmol/L) up to 2 h. Cell lysates were subjected to Western blot and detected with antibodies against phosphorylated p53-Thr^{81} (*p-p53-Thr*^{81}), phosphorylated p53-Ser^{15} (*p-p53-Ser*^{15}), p53, MDM2, and tubulin.

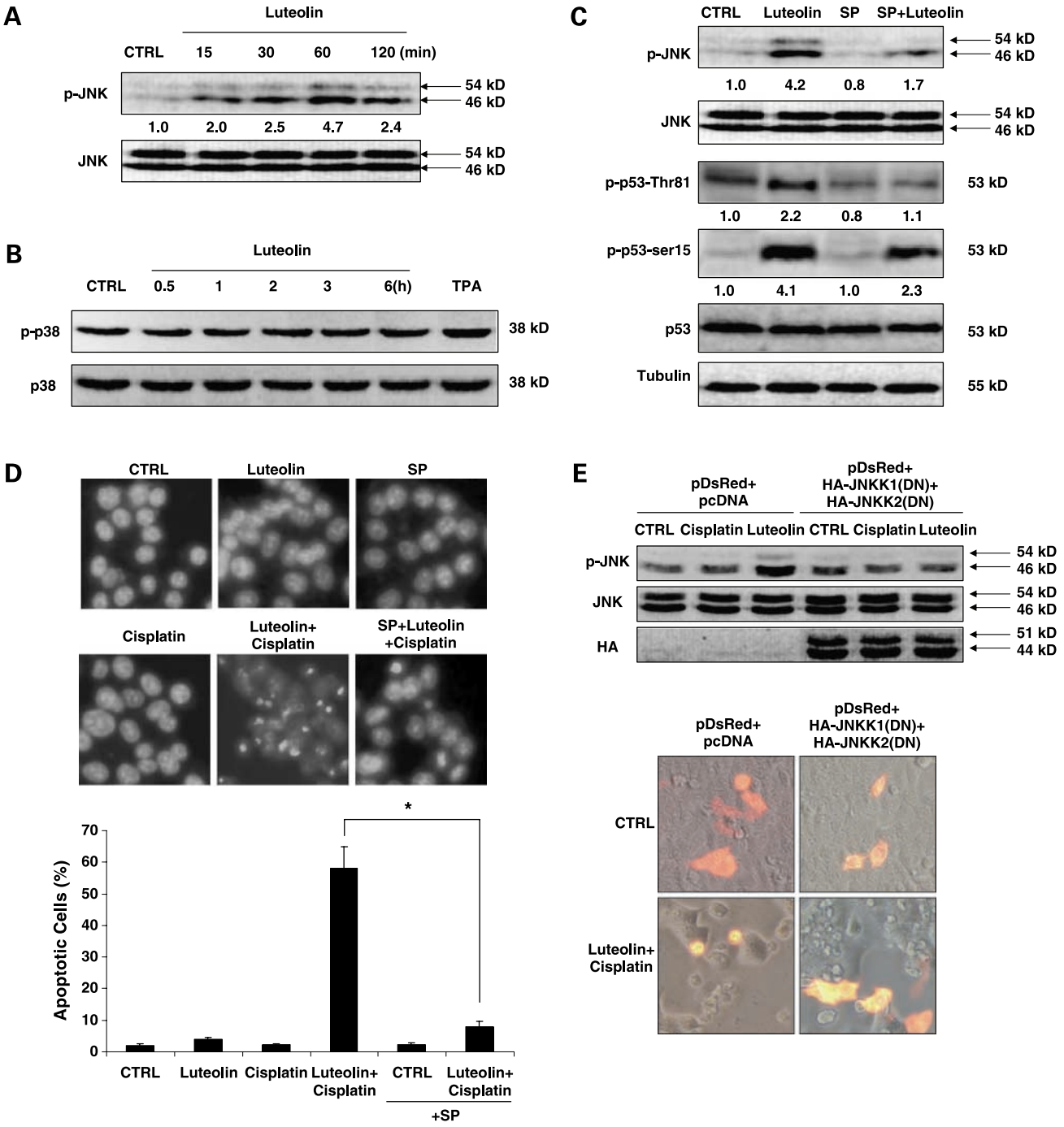

Figure 4. JNK, but not p38, is involved in luteolin-induced increase of p53 phosphorylation and subsequent cell death. **A,** HCT116 cells were treated with luteolin (40 μmol/L) up to 2 h. Cell lysates were subjected to detection of phosphorylated JNK (*p-JNK*) and JNK using Western blot. **B,** cells were treated with luteolin (40 μmol/L) up to 6 h and collected for detection of phosphorylated p38 (*p-p38*) and p38 using Western blot. Cells with TPA (80 nmol/L × 2 h) treatment served as a positive control. **C,** cells were treated with luteolin (40 μmol/L) for 1 h with or without the presence of 1-h pretreatment of SP600125 (20 μmol/L). Cells were collected and subjected to Western blot for detection of phosphorylated JNK, JNK, phosphorylated p53-Thr^{81}, phosphorylated p53-Ser^{15}, p53, and tubulin. **D,** HCT116 cells were pretreated with SP600125 (20 μmol/L ×1 h) followed by treatment of luteolin (40 μmol/L × 2 h). Then cells were treated with cisplatin (10 μg/mL) for another 24 h. Cell death was evaluated using DAPI staining. Apoptotic cells were counted according to their morphologic changes under a fluorescence microscope. *Columns,* mean from three independent experiments; *bars,* SD. **E,** HCT116 cells were transiently transfected with either pcDNA or HA-JNKK1(DN) + HA-JNKK2(DN), together with pDsRed as a transfection marker. Twenty-four hours after transfection, cells were treated with or without luteolin (40 μmol/L) or cisplatin (10 μg/mL) for 1 h and subjected to Western blot for detecting phosphorylated JNK, JNK, and HA-tag. Cell death was evaluated after 2 h pretreatment of luteolin (40 μmol/L) followed by cisplatin (10 μg/mL) for another 24 h. Apoptotic cells were counted according to their morphologic changes under a fluorescence microscope, and those successfully transfected cells were in bright red.

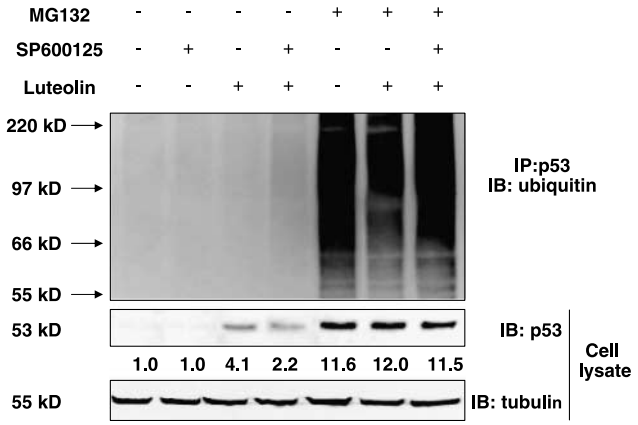

Figure 5. Luteolin decreases p53 ubiquitination through activating JNK. After pretreatment of MG132 (2 μmol/L × 1 h), HCT116 cells were treated with luteolin (40 μmol/L) for 6 h with or without the presence of 1-h pretreatment of SP600125 (20 μmol/L). Cell lysate was used for immunoprecipitation with anti-p53 antibody followed by Western blot with anti-ubiquitin antibody. *IB,* immunoblotting; *IP,* immunoprecipitation.

Altogether, these results strongly suggest that JNK plays a critical role in the cell death induced by luteolin and cisplatin, most probably via p53 phosphorylation and stabilization.

Luteolin Stabilizes p53 via Reduced Protein Ubiquitination and Proteasomal Degradation

It is well known that p53 protein stability is regulated by its phosphorylation and interaction with specific ubiquitin ligases that promote its ubiquitination and proteasomal degradation (11, 38). Here, we further examined whether treatment of luteolin stabilizes p53 through inhibiting p53 ubiquitination in a JNK-dependent manner. As shown in Fig. 5, luteolin treatment for 6 h induced obvious p53 accumulation, which could be suppressed by SP600125. More interestingly, it was found that luteolin treatment significantly decreased the level of ubiquitinated p53 in HCT116 cells in the presence of proteasome inhibitor MG132, whereas the JNK inhibitor SP600125 effectively restored the level of ubiquitinated p53 (Fig. 5). The above results thus clearly show that luteolin stabilizes p53 by suppressing the ubiquitination of p53, a process related to JNK activation.

Luteolin and Cisplatin Induce p53 and Bax Mitochondrial Translocation

Recent evidence suggests that apoptosis mediated by p53 involves p53 mitochondrial relocation (40, 41). To determine the downstream cell death pathways following enhanced p53 protein level in cells with luteolin and cisplatin combined treatment, we examined the level of p53 in isolated mitochondrial fraction. As shown in Fig. 6A, the combined treatment of luteolin and cisplatin caused a significant enhancement of p53 translocation to mitochondria, much higher than that caused by luteolin or cisplatin individual treatment.

p53-mediated transcription-independent apoptosis often requires the involvement of another partner (Bax), and its the proapoptotic function is closely related to its conformational change and mitochondrial translocation (42). In this study, although the Bax total level was not increased by either luteolin or cisplatin or their combination (data not shown), luteolin and cisplatin induced evident Bax mitochondrial translocation (Fig. 6A) and Bax conformational changes as detected by immunoprecipitation using a specific Bax antibody against Bax with conformational change (Fig. 6B). As a result, a significant amount of cytochrome *c* was released from mitochondria (Fig. 6C), thus leading to the activation of the caspase cascade and apoptotic cell death.

Luteolin Enhances the Anticancer Effect of Cisplatin *In vivo*

In this study, we further tested the sensitization effect of luteolin on cisplatin-induced cell killing using a nude mice xenograft model. As shown in Fig. 7A, in the control group, the xenografted HCT116 cells grew rapidly,

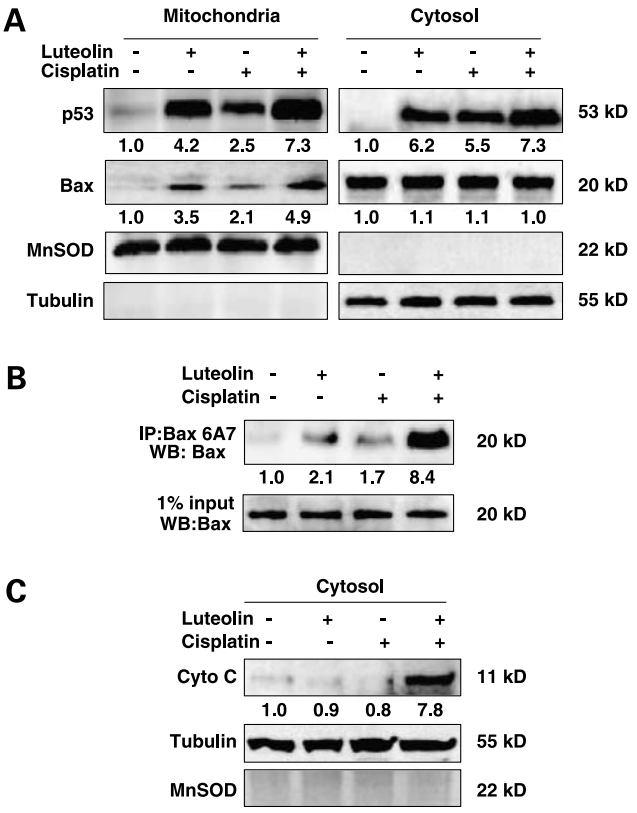

Figure 6. p53 acts on mitochondria to induce apoptotic cell death. **A,** HCT116 cells were treated with luteolin (40 μmol/L) or cisplatin (10 μg/mL) or their combination for 12 h. Mitochondrial and cytosolic fractions were obtained as described in Materials and Methods and subjected to Western blot for the detection of p53, Bax, MnSOD, and tubulin. **B,** cells were treated as described above and cell lysate was used for immunoprecipitation using anti-Bax (6A7) antibody followed by Western blot using anti-Bax antibody. Bax in 1% input lysate was used as a loading control. **C,** HCT116 cells were treated with luteolin (40 μmol/L × 2 h) followed by cisplatin (10 μg/mL) for 18 h. Cells were then lysed and cytosolic fraction was collected for detection of cytochrome *c* (*Cyto C*), MnSOD, and tubulin using Western blot.

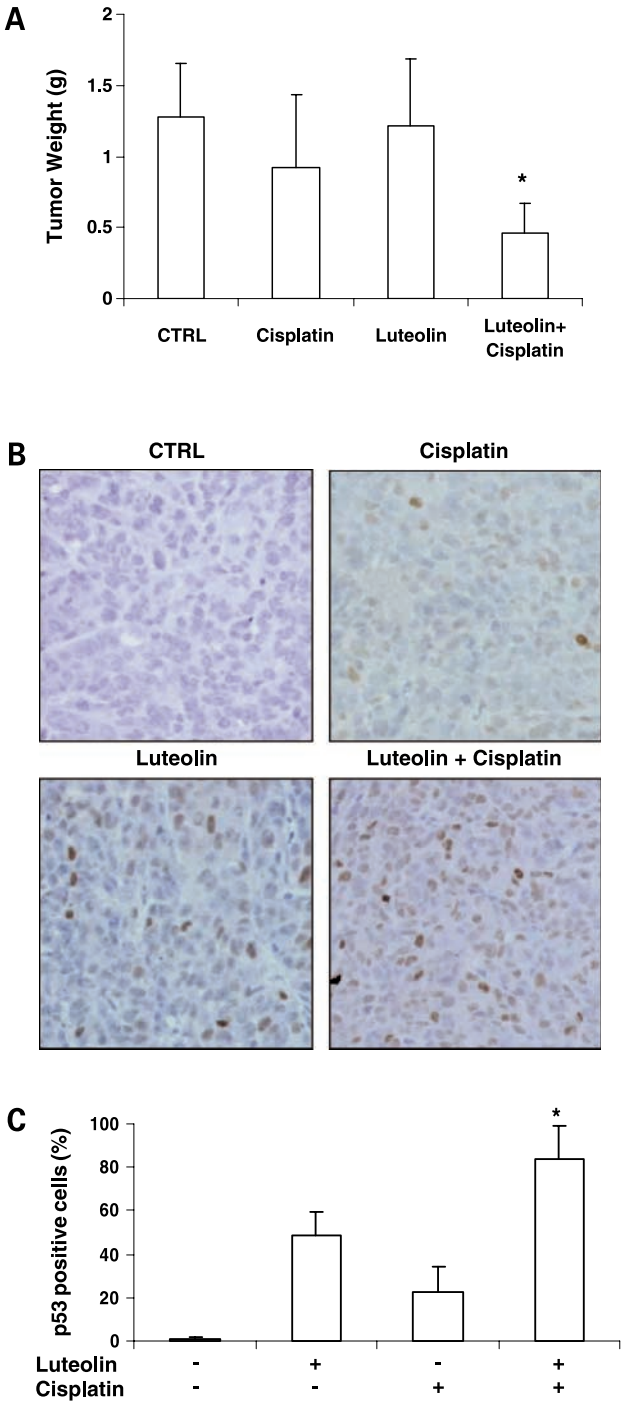

Figure 7. Luteolin enhances the anticancer effect of cisplatin through elevating p53 protein *in vivo*. **A,** BALB/c nude mice were inoculated with HCT116 cells as described in Materials and Methods. At the end of treatment, mice were sacrificed by CO_2 inhalation and photographed. Tumor weights (g) of mice were measured. *Columns,* mean (n = 12); *bars,* SD. **B,** p53 expression level was evaluated by immunohistochemistry in tumor tissues. The representative field for p53 expression was photographed (×200). **C,** percentages of p53-positive cells were counted in representative fields including at least 200 cells. *Columns,* mean (n = 12); *bars,* SD. *, $P < 0.05$, compared with the control group.

forming a tumor weighting an average about 1.28 g in 3 weeks. Cisplatin (1.25 mg/kg body weight) alone or luteolin (40 mg/kg body weight) alone failed to induce significant suppression of tumor growth, whereas the combined treatment of luteolin and cisplatin markedly reduced the tumor weight to 0.46 g. To explore the underlying mechanisms, the p53 protein level was determined in tumor tissues using immunohistochemistry. As shown in Fig. 7B and C, a very low level of p53 was observed in the control group. In contrast, about 50% of the cells were found to be p53 positive in animals receiving luteolin alone, whereas >80% cancer cells were p53 positive in the mice treated with a combination of luteolin and cisplatin. Such findings are basically consistent with the observations in cell culture experiments *in vitro* (Fig. 2A) and support the notion that luteolin enhances the therapeutic activity of cisplatin via up-regulation of p53 protein level.

Discussion

Cisplatin is one of the most commonly used cancer therapeutic agents. However, resistance to this drug is a major limitation for its clinical use (3, 27). Therefore, combined treatment with other sensitizing agents is an effective strategy to overcome cisplatin resistance (43–46). Here, we provide experimental both *in vivo* and *in vitro* evidence that luteolin, a natural flavonoid, is able to enhance the therapeutic potential of cisplatin. In this study, we first confirmed the critical role of p53 in cell death induced by combined treatment of luteolin and cisplatin, based on the following observations: (*a*) evident apoptotic cell death was only observed in p53 wild-type cancer cells, but not in p53 mutant cells (Fig. 2B); (*b*) knockdown of p53 offered significant protection against apoptosis induced by luteolin and cisplatin (Fig. 2C and D).

The tumor suppressor p53 is well known to be an important regulator in cancer cell apoptosis in response to cancer therapeutics, such as cisplatin (1, 3, 4). One important finding from this study is that luteolin or cisplatin is able to significantly enhance intracellular p53 protein level (Fig. 2A). However, there are two fundamental differences between the effect of luteolin and cisplatin: (*a*) luteolin treatment leads to a more rapid increase of p53 protein level than cisplatin (Fig. 2A); (*b*) cisplatin, but not luteolin, increases p53 mRNA level detected by reverse transcription-PCR (data not shown). Such differences indicate that luteolin or cisplatin acts on p53 via distinctive mechanisms. It has been reported that luteolin can activate p53, without knowing the molecular mechanisms involved (47). Data from this study suggest that luteolin-induced p53 up-regulation is achieved at the posttranscriptional level via enhanced p53 protein stabilization. Our conclusion that luteolin sensitizes cisplatin-induced apoptosis via p53 stabilization is also supported by data from the *in vivo* study. Immunohistochemistry staining showed the synergistic effect of luteolin and cisplatin in enhancing the p53 protein level in the tumor tissue (Fig. 7B and C), which is

consistent with the *in vitro* data (Fig. 2A). It is thus believed that luteolin is capable of enhancing cisplatin-mediated cancer cell apoptosis via activating p53-dependent pathways.

After confirming the role of p53 in luteolin- and cisplatin-induced apoptotic cell death, we next focused on the molecular mechanisms by which luteolin stabilizes p53 protein level. It is well established that p53 stability is tightly controlled by MDM2, and the p53-MDM2 interaction is in turn largely regulated by the phosphorylation status of p53 (10, 12, 48). In this study, MDM2 itself may not be the target for luteolin because neither the MDM2 protein level (Fig. 3B) nor the MDM2 and p53 protein interaction was affected by luteolin (data not shown). Instead, it is likely that luteolin stabilizes p53 via JNK-mediated protein phosphorylation. First, luteolin increased the p53 phosphorylation at Thr^{81} and Ser^{15} (Fig. 3B), a profile consistent with the phosphorylation specificity of JNK (39). Second, activated JNK, but not p38, was found in cells treated with luteolin (Fig. 4A and B). Third, SP600125, a specific JNK inhibitor, suppressed luteolin-induced JNK activation and thus abolished p53 phosphorylation (Fig. 4C). Notably, JNK has been reported to stabilize p53 in MDM2-null cells (49, 50). Thus, luteolin might activate JNK and phosphorylate and stabilize p53 through a MDM2-independent mechanism.

Thus far, the reports on the effect of JNK on p53 protein phosphorylation and ubiquitination are not consistent. It has been reported that JNK is able to destabilize p53 by promoting ubiquitin-mediated degradation in nonstressed cells (49). Conversely, a variety of stress factors often activate JNK, which subsequently phosphorylates p53, suppresses ubiquitin-mediated protein degradation, and thus stabilizes the p53 protein (50). It is thus believed that the relation between JNK and p53 might depend on whether cells are under stress status. In the present study, we observed a decrease in ubiquitinated p53 level after luteolin treatment, which can be largely recovered by SP600125 (Fig. 5). It is thus possible that luteolin treatment might serve as a cellular stress factor, leading to the enhanced p53 protein phosphorylation, subsequently reduced p53 ubiquitination and proteasomal degradation, and eventually the accumulation of intracellular p53 protein via JNK activation. As a result, higher level of p53 then attributes to enhanced cell death induced by luteolin in cisplatin-treated cancer cells. Such a notion is clearly supported by the findings that inhibition of JNK using either pharmacologic or genetic approaches is able to diminish cell death induced by combined treatment of luteolin and cisplatin (Fig. 4D and E).

One important role of p53 as a tumor suppressor is its involvement in apoptosis via an intrinsic pathway that involves mitochondria, a central regulator of apoptosis. This process is known to be mediated by p53-controlled *de novo* transcription of some proapoptotic proteins, such as Bax, Noxa, and PUMA (51–53), or via p53 mitochondrial translocation (40, 41). Activation of p53, as well as other death stimuli, can perturb the balance of Bcl-2 family proteins and cause release of proapoptotic proteins such as cytochrome *c* from mitochondria to cytosol. In this study, luteolin is capable of inducing significant p53/Bax mitochondrial translocation and Bax transformation, suggesting that the elevated p53 executes its proapoptotic function via the mitochondrial pathway.

In summary, luteolin, an important flavonoid, can significantly enhance the anticancer effect of cisplatin both *in vivo* and *in vitro*. Luteolin is able to stabilize p53 protein via increasing JNK activation and p53 protein phosphorylation, leading to reduced p53 ubiquitination and proteasomal degradation. Based on the critical role of p53 in controlling the cell death processes in response to various cancer therapies, we postulate that luteolin may act as a potent chemosensitizer, especially in cancer with wild-type p53. In fact, we have observed similar sensitization effect of luteolin on other cancer chemotherapeutic agents, such as Adriamycin and etoposide.[4] Although further investigations are needed to elucidate the underlying mechanisms of luteolin on enhancing the anticancer effects of chemotherapeutic agents, data from this study provide novel evidence for the potential clinical application for luteolin as a chemosensitizer in cancer therapy.

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