Available online at www.sciencedirect.com

SCIENCE DIRECT

European Journal of Pharmacology 502 (2004) 169–183

www.elsevier.com/locate/ejphar

Extracellular signal-regulated kinase activation and Bcl-2 downregulation mediate apoptosis after gemcitabine treatment partly via a p53-independent pathway

Gee-Chen Chang[a,b,*], Shih-Lan Hsu[b,c], Jia-Rong Tsai[a], Wen-Jun Wu[b], Chih-Yi Chen[d], Gwo-Tarng Sheu[b]

[a]*Division of Chest Medicine, Department of Internal Medicine, Taichung Veterans General Hospital, Taichung, Taiwan*
[b]*Institute of Toxicology, Chung Shan Medical University, Taichung, Taiwan*
[c]*Department of Education and Research, Taichung Veterans General Hospital, Taichung, Taiwan*
[d]*Division of Thoracic Surgery, Taichung Veterans General Hospital, Taichung, Taiwan*

Received 12 July 2004; received in revised form 30 August 2004; accepted 1 September 2004
Available online 27 September 2004

Abstract

Gemcitabine is a promising compound for the treatment of human lung cancer. Although apoptosis has been shown to play a role in certain cell types with gemcitabine, the steps leading to cell death after the drug–target interaction are not well understood. We studied the molecular mechanisms of gemcitabine-induced apoptosis and determined the role of p53 function on the cytotoxic effects of gemcitabine in human nonsmall cell lung cancer (NSCLC) H1299 and H1299/p53 cells. Here, we found that gemcitabine induced an apoptotic cell death via a Bcl-2-dependent caspase-9 activation pathway. Moreover, phosphorylated activation of extracellular signal-regulated kinase (ERK) was observed upon gemcitabine treatment. Genetical or pharmacological inhibition of ERK activation markedly blocked gemcitabine-induced cell death. Furthermore, inactivation of Akt was also involved in this event. Taken together, our observations indicate that ERK activation and Akt inactivation mediated gemcitabine-induced apoptosis independently of p53 in human NSCLC H1299 cells.

Keywords: Akt; Apoptosis; Bcl-2; ERK; Gemcitabine; p53

1. Introduction

Lung cancer remains the leading cause death in the world, both in men and women. The poor lung cancer survival rates argue powerfully for effective approaches to control this disease. Gemcitabine (2′,2′-difluoro-2′-deoxycytidine), a nucleoside analogue, is structurally related to deoxycytidine with two fluorine substitutes for the two hydrogen atoms in the 2′ position of the deoxyribose sugar and has been demonstrated to be one of the most active chemotherapeutic agents in several solid tumors, especially nonsmall cell lung cancer (NSCLC) (Bunn and Kelly, 1998). Gemcitabine enters the cell via a nucleoside transport system (Graham et al., 2000; Mackey et al., 1998) and is activated to its difluorodeoxycytidine triphosphate, which is incorporated into DNA or RNA, consequently leading to chain termination. These are potentially important mechanisms of action of this drug. Although the cytotoxic action of gemcitabine has been shown to be correlated with the amount of drug incorporated into DNA and RNA (Nabhan et al., 2001), the molecular pathway by which this drug caused cell death has not been fully defined.

The signaling events implicated in survival, growth arrest, or programmed cell death in response to DNA-damaging stress include the activation of mitogen-acti-

* Corresponding author. Division of Chest Medicine, Department of Internal Medicine, Taichung Veterans General Hospital, No. 160, Section 3, Chung-Gang Road, Taichung, Taiwan, ROC. Tel.: +886 4 23592525x3201, 3215, 3218; fax: +886 4 23500034.
E-mail address: august@vghtc.gov.tw (G.-C. Chang).

0014-2999/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2004.09.006

vated protein kinase (MAPK) pathways (Tang et al., 2002). Three major mammalian MAPK superfamilies have been identified: the extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK; also called stress-activated protein kinase), and p38 kinases. ERK5 is the largest known MAPK family member (about 100 kDa); the core catalytic domain is at its N-terminus and is 51% identical to ERK2. Each MAPK is activated through a specific phosphorylation cascade (Chen et al., 2001). The importance of MAPK signaling pathways in regulating apoptosis during conditions of stress has been widely investigated. Previous studies have demonstrated that activation of the ERK pathway delivers a survival signal that counteracts proapoptotic effects associated with JNK and p38 activation (Xia et al., 1995; Chen et al., 1996; Graves et al., 1996; Brenner et al., 1997). However, ERK activation plays an active role in mediating DNA-damaging drug-induced apoptosis and functions upstream of caspase activation to initiate the apoptotic signal (Wang et al., 2000; Tang et al., 2002; Woessmann et al., 2002). There is also mixed evidence for the role of MAPK in influencing the cell survival of chemotherapeutic drug-treated cells. Several studies have highlighted the cross-talk between the MAPK pathway and the PI3K–Akt signaling cascade (Shelton et al., 2003). PI3K functions in multiple signal transduction pathways by interacting with oncogenes, which leads to cellular transformation in ovarian, breast, and nonsmall cell lung cancers (Stambolic et al., 1999). The amplification or upregulation of PI3K–Akt signaling results in the development of cancer; thus, targeting and downregulating PI3K or Akt activity are critical for cancer therapy. Whether gemcitabine induced signaling events leading to the activation of MAPK and its downstream targets or inactivation of Akt signaling remains unclear. In the present study, the roles of Akt, MAPK, and its downstream transcription factor nuclear factor-kappa B (NF-kB), and caspase cascade in regulating gemcitabine-induced apoptosis were examined in human NSCLC H1299 cells.

p53, a nuclear phosphoprotein, plays an important role in apoptosis, growth arrest, genomic stability, cell senescence, and differentiation. *p53* mutations are the most common genetic changes found in human cancer, and mutations in this gene result in loss of its function and inactivation (Hollstein et al., 1991; Ko and Prives, 1996), suggesting its pivotal role in human carcinogenesis and outcome of treatment. Numerous studies have identified mutations of the *p53* gene in lung cancer (p53 is mutated in the majority of small cell lung cancers and in over 50% of NSCLC) and have shown a correlation of p53 status with disease progression and prognosis (Chiba et al., 1990). Characterizing how the presence of wild-type functional p53 compared with functionally mutated p53 affects the susceptibility of lung cancer to specific chemotherapeutic compounds could impact clinical decisions regarding adjuvant or therapeutic use of such drugs. However, the effect of p53 alterations and response to chemotherapy is complex. Cellular induction of apoptosis clearly does not occur by a single p53-dependent pathway. In addition, chemotherapeutic agents have varying mechanisms of action. With increasing interest in the clinical significance of *p53* mutation for managing lung cancer therapy, a better definition of drug activity in *p53*-mutated and wild-type lung cancers could help direct clinical studies and applications. Although the in vitro activity of gemcitabine against NSCLC has been demonstrated previously (Pace et al., 2000), the relationship between gemcitabine sensitivity and p53 status in NSCLC remains controversial. In support of a role for p53 in the cytotoxic mechanism of gemcitabine, several studies demonstrated that disruption of p53 function afforded drug resistance (Chen et al., 2000; Achanta et al., 2001; Galmarini et al., 2002). Conversely, cell lines that do not exhibit a predominant apoptotic response to gemcitabine were sensitized to gemcitabine by the inactivation of p53 (Kielb et al., 2001). Therefore, another aim of this study was to characterize the effects of gemcitabine on the distribution of the cell cycle and the expression of apoptosis-related genes in human lung cancer cell line with p53-null and wild-type p53. We transfected a wild-type *p53* gene into human NSCLC H1299 cells, in which p53 is homozygously deleted (Nguyen et al., 1996), to investigate whether ectopic expression of p53 could modulate the apoptosis induced by gemcitabine and to determine the role of p53 in the downstream biochemical signals, cell cycle arrest, and cell death induced by this drug. Our results clearly show that ERK-dependent Bcl-2 down-regulation and caspase activation were involved in gemcitabine-induced apoptosis. In addition, elimination of the survival pathway of AKT per se may be partly a lethal condition for gemcitabine treatment. Whereas p53 is an important factor in the short-term response to drug treatment, wild-type p53 function is not an absolute requirement for gemcitabine-induced cytotoxicity.

2. Materials and methods

2.1. Materials

Gemcitabine was provided by Eli Lilly (Indianapolis, IN, USA). Anti-ERK1/2, anti-ERK1/2 phospho-specific antibodies, and 2-[4-morpholinyl]-8-phenyl-4*H*-1- benzopyran-4-one (LY294002) were purchased from Calbiochem Chemical (CN Biosciences Notts, UK). Anti-Bcl-X_L and anti-Bad antibodies were purchased from Transduction Laboratory. Anti-$p27^{KIP1}$, anti-Bcl-2, anti-Bax, and anti-Bak antibodies were obtained from Santa Cruz Biotechnologies. Anticytochrome *c*, anti-$p21^{CIP1/WAF1}$, and anti-p53 antibodies were obtained from Upstate Biotechnology. Anti-phospho-Akt antibodies and 1,4-diamino-2,3-

dicyano-1,4-bis[2-amino phenylthio]-butadiene (U0126) were purchased from Cell Signaling Technology (Beverly, MA, USA). 2-[2-Amino-3-methoxyphenyl]-4*H*-1-benzopyran-4-one (PD98059) and ammonium pyrrolidinedinedithiocarbamate (PDTC) were obtained from Sigma (St. Louis, MO, USA). The inhibitors of caspase-3 (Z-DEVD-FMK), caspase-8 (Z-IETD-FMK), and caspase-9 (Z-LEHD-FMK) were obtained from Kamiya (Thousand Oaks, CA, USA). The expression plasmid for MAP kinase phosphatase (MKP3) in the pSG5 vector was provided by J. Pouyssegur (Universite de Nice, Nice, France).

2.2. Cell culture and p53 plasmid transfection

H1299 cells (a human nonsmall cell lung carcinoma cell line that contains a homozygous deletion of the *p53* gene) (Nguyen et al., 1996) were obtained from the American Type Culture Collection (Rockville, MD, USA) and maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum in a 37 °C incubator containing 5% carbon dioxide. H1299 cells were transfected by pC53SN plasmids, which were kindly given by Dr. W.H. Lee. Expression plasmid was the derivative of pCDNA3 (Invitrogen) constructed by ligation of a 1192-bp *Eco*RI and *Xba*I fragment derived from pC53SN by polymerase chain reaction. Primer A (GGAATTCATGGAGGAGCCGCAGTCAGATCC) and primer B (AGTCTAGATCAGTCTGAGTCAGGCCCTTCTG) contained the entire p53 coding sequence in frame and were used to insert the fragment into the *Eco*RI/*Xba*I sites of pCDNA3. Stable clones were selected by G418 treatment. In addition, expression of exogenous p53 was also conducted by adenovirus p53 construct and was verified by immunoprecipitation with specific anti-p53 antibody.

2.3. Growth inhibition and apoptotic cell determination

Cells were seeded into 12-well plates at 3×10^4 cell/well. After 24 h, cells were treated with various concentration of gemcitabine (0.01, 0.1, and 1 μM) for the indicated time points. Cell numbers were determined by Trypan blue dye exclusion, using a hemocytometer. Apoptotic cells were determined by in situ terminal transferase-mediated dUTP fluorescein nick end-labeling (TUNEL) assay (Boehringer Mannheim, Roche Applied Science). Cells were seeded at a density of 3×10^4 cells/well onto a 12-well plate 24 h prior to drug treatment. Gemcitabine was added to the medium at various concentrations and times. For in situ TUNEL assay, cells were fixed with 80% ethanol or 2% paraformaldehyde at room temperature for 30 min and permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (PBS), then exposed to terminal transferase reaction mixture (34 mU/ml terminal transferase, 280 pmol of dATP, 90 pmol of fluorescein-11 dUTP, 30 mM Tris–HCl, 140 mM sodium cacodylate, 1 mM $CoCl_2$, pH 7.2) for 1 h at 37 °C in the dark. Cells were subsequently washed with PBS and assessed using a flow cytometer (Becton Dickinson Instruments) or examined using a fluorescence microscope.

2.4. Cell cycle analysis

Cells were treated with or without gemcitabine for the indicated time periods, and cell cycle distribution was analyzed using flow cytometry. Briefly, 1×10^6 cells were trypsinized, washed with PBS, and fixed in 80% ethanol; then washed with PBS, incubated with 100 μg/ml RNase at 37 °C for 30 min, stained with propidium iodide (50 μg/ml), and analyzed on a FACScan flow cytometer. The percentage of cells in different phases of the cell cycle was analyzed using Cell-FIT software (Becton Dickinson Instruments).

2.5. Measurement of caspase activity

Caspase activity was measured according to the manufacturer's protocol (R&D Systems). Briefly, cell lysates (100 μg of total protein) were added to reaction mixtures (final volume 50 μl) containing fluorogenic substrate peptides specific for caspase-3 (DEVD-AFC), caspase-8 (IETD-AFC), and caspase-9 (LEHD-AFC). The reaction was performed at 37 °C for 2 h. Fluorescence was measured with a fluorescence microplate reader (Thermo Labsystem, Finland) (excitation wavelength 400 nm, emission wavelength 505 nm).

2.6. Protein preparation an immunoblotting

H1299 and H1299/p53 cells were cultured without or with 1 μM gemcitabine for the indicated time points. After treatment, cell extracts were prepared and protein concentrations were determined using the Bradford method. Equal amounts of sample lysates were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting with specific primary antibodies, including anti-Bcl-2, Bcl-X_L, Bax, Bak, Bad, p53, $p21^{CIP1/WAF1}$, $p27^{KIP1}$, ERK, and phospho-ERK antibodies. Determinations were made using enhanced chemiluminescence kits (ECL kit; Amersham).

2.7. MEK inhibition assay

H1299 cells were cultured with MEK inhibitor (20 μM U0126 or 40 μM PD98059) for 2 h in dark. Then 1 μM gemcitabine was added for indicated time periods. Cell numbers were determined by Trypan blue dye exclusion, using a hemocytometer. Or apoptotic cell number was measured by TUNEL assay. Cell extracts were prepared and protein concentrations were determined using the Bradford method. Equal amounts of sample lysates were separated by SDS-PAGE and immunoblotting with primary antibodies,

including anti-Bcl-2, Bcl-X_L, ERK, and phospho-ERK antibodies.

2.8. Transient transfection analysis

H1299 and H1299/p53 cells were transiently transfected with MKP3-pSG5 vector and control vector by the lipofectamine method according to the manufacturer's protocol (Invitrogen, Life Technologies). After transfection for 24 h, cells were treated with or without 1 μM gemcitabine for another 72 h, apoptotic cells were determined, and cell lysates were prepared for the detection of phosphorylated ERK.

2.9. Electrophoretic mobility shift assay

The DNA-binding assay for the detection of activated AP-1 and NF-κB was performed as described elsewhere (Berger et al., 1993; Rodgers et al., 2000). Nuclear extracts were prepared from untreated or gemcitabine-treated H1299 or H1299/p53 cells for the indicated time periods. Specific oligonucleotides containing consensus DNA-binding site for AP-1 (5′-biotin-CGCTTGATGAGTCAGCCGGAA-3′ and 5′-biotin-TTCCGGCTGACTC ATCAAGCG-3′) and NF-κB (5′-biotin-AGTTGAGGGGACTTTCCCAGGC-3′ and 5′-biotin-GCCTGGGAAAGTCCCCTCAACT-3′), respectively. The single-stranded sense and antisense were boiled and annealed to generate a double-stranded oligonucleotide. DNA binding was performed at 30 °C for 20 min in a final volume of 20 μl, which contained 5 μg of nuclear extract, 5 pmol of biotin-labeled NF-κB or AP-1-specific consensus oligonucleotide, 20 μg of poly(dI/dC) (Pharmacia, Freiburg, Germany), 2 μl of buffer A (20 mM HEPES, pH 7.9, 20% glycerol, 100 mM KCl, 0.5 mM EDTA, 0.25% NP-40, 2 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride), 4 μl of buffer B [20% Ficoll 400 (Pharmacia), 100 mM HEPES, pH 7.9, 300 mM KCl, 10 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride]. The DNA–protein complexes were separated in a 6% nondenaturing polyacrylamide gel and transferred onto nitrocellulose membrane. Subsequently, the membrane was fixed with UV light and blocked with 5% nonfat milk. Streptavidin–horseradish peroxidase conjugate was added at 4 °C overnight. Activation of AP-1 and NF-κB was detected using Supersignal Chemiluminescent substrate kits (Pierce, Rockford, USA).

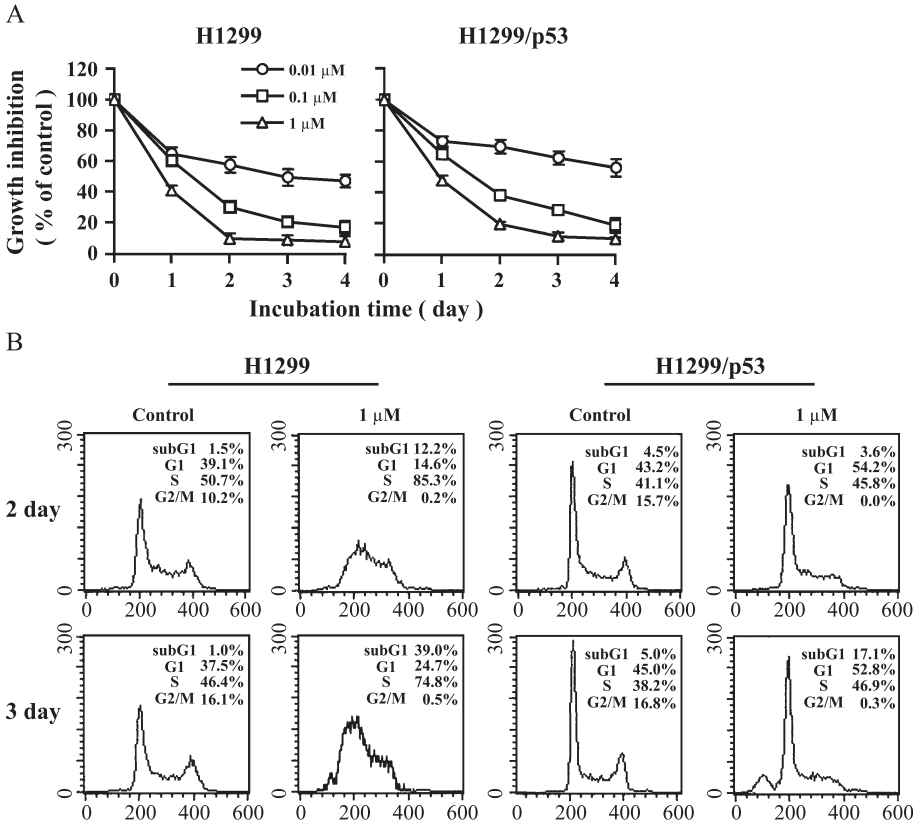

Fig. 1. Gemcitabine-induced apoptosis in H1299 cells. (A) Both H1299 and H1299/p53 cells were treated with various doses of gemcitabine (0.01, 0.1, and 1 μM) for 1–4 days as indicated in the figure. The cells were then counted for their survival rate by Trypan blue dye exclusion method. (B) Flow cytometry analysis of H1299 cells treated with or without 1 μM gemcitabine for 2 or 3 days. (C) Regulation of p53, $p21^{CIP1/WAF1}$, and $p27^{KIP1}$ by gemcitabine. Cells were treated with or without 1 μM gemcitabine for 1, 2, or 3 days. The expression levels of p53, $p21^{CIP1/WAF1}$, and $p27^{KIP1}$ were detected by Western blot analysis. (D) Induction of apoptosis. Cells were treated with or without 1 μM gemcitabine for 3 days. Apoptotic cells were determined by DAPI staining and TUNEL assay. Scale bar, 20 μm.

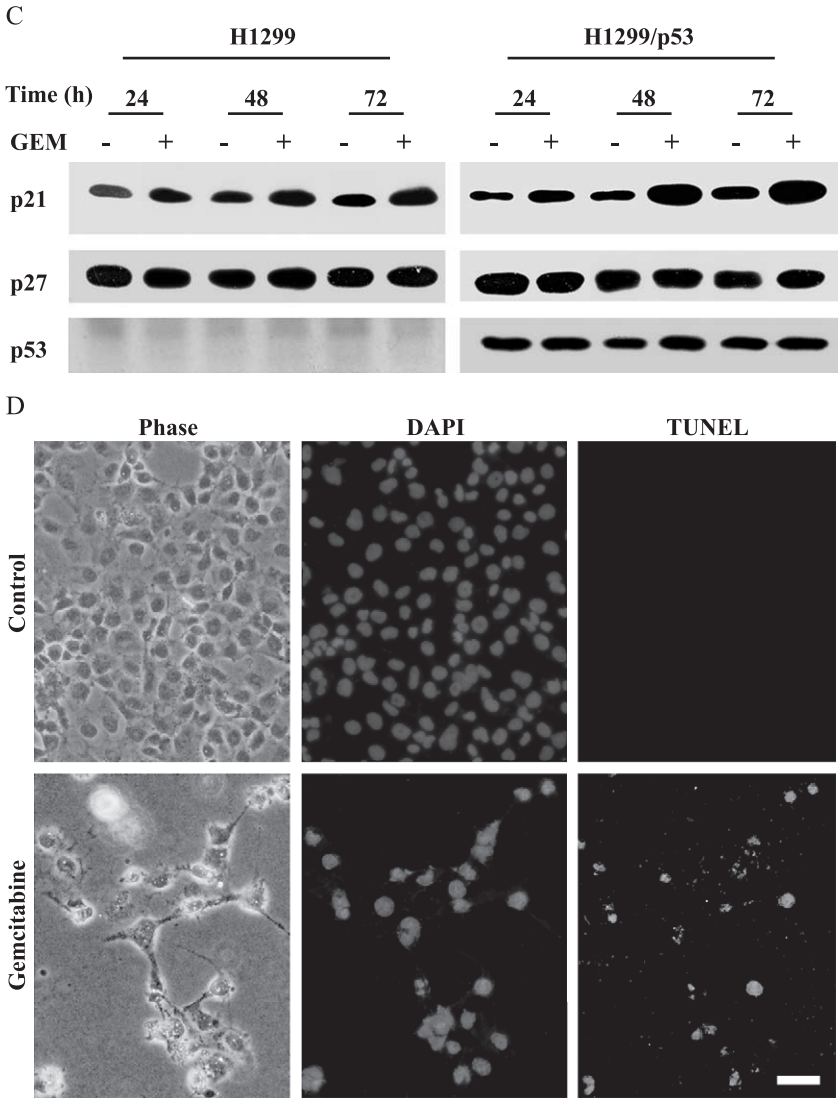

Fig. 1 (*continued*).

2.10. Analysis of data

All data are presented in this study as mean±S.D. of 12 replicates from four separate experiments. For comparisons of two groups, a *t* test for independent samples was used and considered significant at the $*p<0.05$, $**p<0.01$, or $***p<0.001$ levels. All the figures shown in this article were obtained from at least four independent experiments with a similar pattern.

3. Results

3.1. Gemcitabine-induced apoptosis in H1299 and H1299/p53 cells

The effects of gemcitabine on the cell viability of H1299 (p53 null) and H1299/p53 were examined. Cells were exposed to various concentrations of gemcitabine for indicated time points (0–4 days). The results showed that gemcitabine exhibited the antiproliferative effects in a dose-dependent manner in both p53-null and wild-type p53 cell lines (Fig. 1A). It seemed that the p53-null cells were slightly sensitive than the wild-type p53-expressed cells in this assay, especially at a low concentration (0.01 μM) of gemcitabine treatment. Flow cytometry analysis indicated that administration of 1 μM gemcitabine to H1299 caused S-phase arrest (with approximately 85% of cells in S-phase for 2-day treatment) and subsequently resulted in apoptosis in a time-dependent increase in the proportion of cells in the sub-G1 phase (Fig. 1B). In contrast, cell cycle analysis revealed that the gemcitabine-treated H1299/p53 cells accumulated in the both G1- and S-phases of the cell cycle (Fig. 1B), with approximately 54% and 46%, respectively. Western blot analysis showed that the $p21^{CIP1/WAF1}$ protein was markedly induced in gemcitabine-treated H1299/p53 cells but slightly induced in gemcitabine-treated H1299 cells (Fig. 1C), providing a

possible explanation for the observed G1 arrest of H1299/p53 cells. However, the induction of p53 by gemcitabine in H1299/p53 cells could not be detected. The level of p53 protein was constantly expressed in untreated or gemcitabine-treated H1299/p53 cells.

In addition, treatment of H1299 cells with 1 μM gemcitabine resulted in progressive morphological changes typical of apoptosis, including cell shrinkage, rounding, and detachment of the cells from the plate, as observed with phase contrast microscopy (Fig. 1D). Examination of these cells under a fluorescent microscope after TUNEL assay and DAPI staining showed characteristic chromatin condensation and nuclear fragmentation into clearly visible apoptotic bodies in gemcitabine-treated H1299 cells (Fig. 1D). There were no typical morphological features of apoptosis found in the control groups. Similar results were obtained with TUNEL assay and DAPI staining of the H1299/p53 cells (data not shown). Moreover, treatment with 1 μM gemcitabine caused a direct apoptotic effect on tested cells—a significantly higher number of apoptotic cells (subG1 population) in the p53-null cells than in wild-type p53-expressed cells (Fig. 1B).

3.2. Gemcitabine-induced apoptosis through mitochondrial cellular execution pathway

It has been well documented that caspase activation was important for the execution phase of apoptosis (Cohen, 1997). We therefore examined the activation of caspases in cells treated with 1 μM gemcitabine. Data from caspase

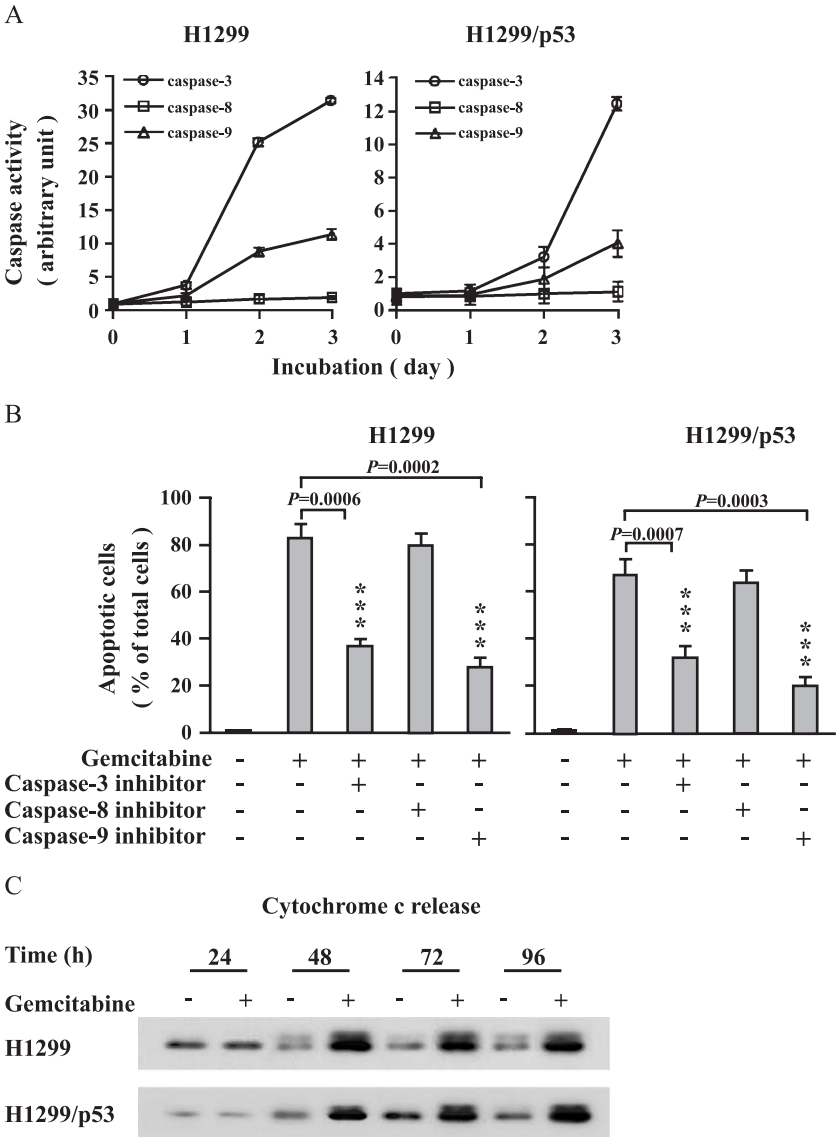

Fig. 2. Gemcitabine-induced caspase activation and cytochrome *c* release. H1299 and H1299/p53 cells were treated with 1 μM gemcitabine for various time points followed by (A) assay of specific caspase activities. (B) Prevention of gemcitabine-induced apoptosis by caspase inhibitors. Cells were treated with 1 μM gemcitabine in the presence or absence of caspase inhibitors for 3 days. Apoptotic cells were determined by TUNEL assay using flow cytometer. (C) The level of cytosolic cytochrome *c* was measured as described in Materials and Methods.

activity analysis indicated that caspase-3 and caspase-9, but not caspase-8, were activated upon gemcitabine treatment (Fig. 2A). The inhibitor of caspase-9 or caspase-3, but not caspase-8, could markedly block the gemcitabine-triggered apoptosis (Fig. 2B). Based on our observations, the activation of caspase-9 suggests that mitochondrial apoptotic pathway might be involved in gemcitabine-induced cell death in H1299 cell lines. Next, the subcellular location of cytochrome *c*, which normally resides in the mitochondria but is released into the cytoplasm following exposure of cells to certain apoptotic stimuli, has been suggested to participate in activating the cell death process (Joza et al., 2002). As shown in Fig. 2C, addition of gemcitabine to H1299 and H1299/p53 cells caused the accumulation of cytochrome *c* in the cytosolic fraction.

Bcl-2 and related proteins are important regulators of mitochondrial-mediated apoptosis (Joza et al., 2002). To provide a mechanistic view of how gemcitabine-induced H1299 underwent apoptosis, a panel of apoptosis and antiapoptosis-related Bcl-2 molecules was tested for their protein expression as determined by Western blotting. Fig. 3A showed that a significant decrease in Bcl-2 and Bcl-

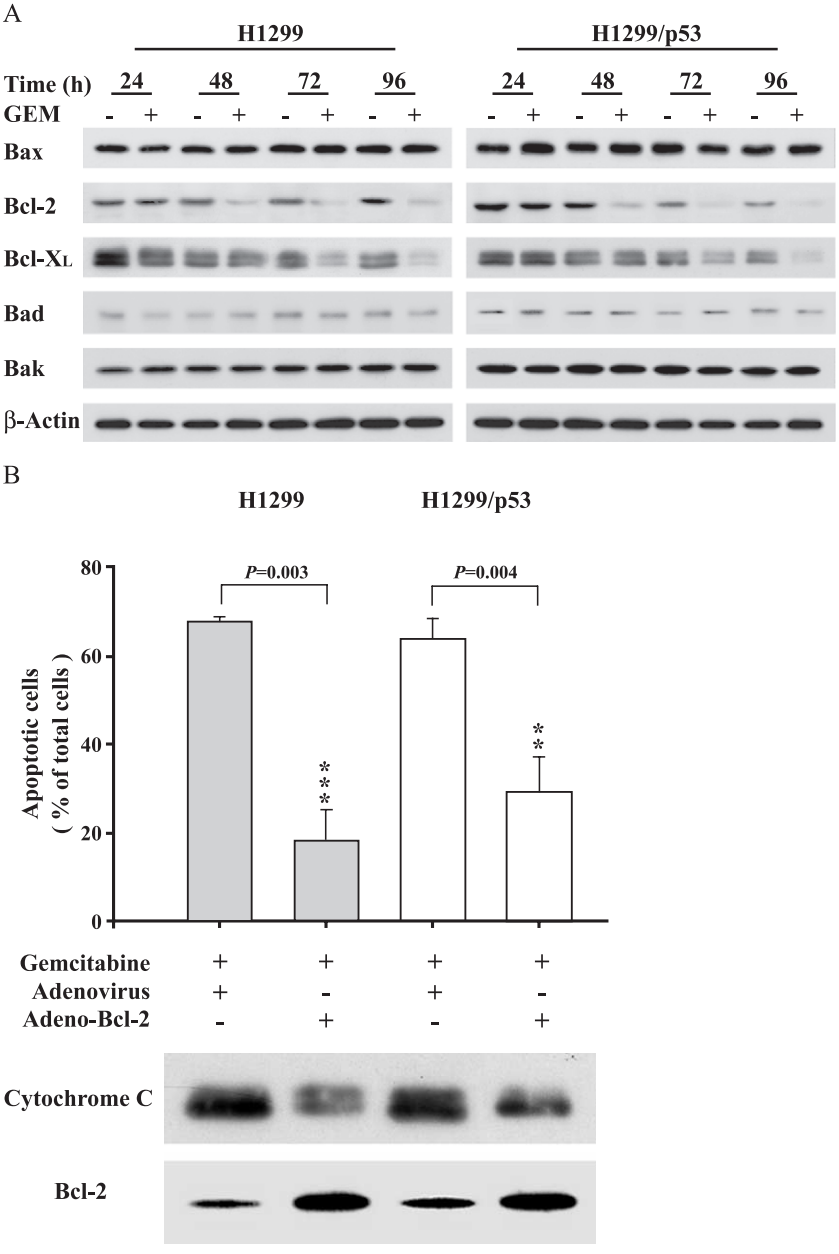

Fig. 3. Involvement of Bcl-2 in gemcitabine-induced apoptosis. (A) H1299 and H1299/p53 cells were treated with 1 μM gemcitabine for indicated time periods (24, 48, 72, and 96 h). After treatment, the protein expression profiles of several apoptotic and antiapoptotic Bcl-2-related proteins were analyzed by Western blot using specific antibodies. (B) Overexpression of Bcl-2-protected cells from gemcitabine-induced apoptosis. Cells were transfected with adeno-Bcl-2 vector. After 8 h, 1 μM gemcitabine was then added to the cells, which were further cultured for 3 days. The levels of expressed Bcl-2 and cytosolic cytochrome *c* were determined by Western blot, and the apoptotic cells were measured using TUNEL assay by flow cytometry.

X_L protein level was observed in H1299 and H1299/p53 cells treated with gemcitabine for 48, 72, and 96 h, as compared with control cultures, whereas there were no significant differences in the protein expression level of Bax, Bad, and Bak. Since treatment of H1299 cells with gemcitabine reduced the Bcl-2 protein level, this observation prompted us to examine the role of Bcl-2 in gemcitabine-induced apoptosis in these cells. Both cell lines were infected with adenovirus Bcl-2 construct and then were exposed to 1 μM gemcitabine. Ectopic expression of Bcl-2 proteins in H1299 and H1299/p53 cells was confirmed by Western blot analysis with specific anti-Bcl-2 antibody (Fig. 3B). Moreover, overexpression of Bcl-2 partially prevented H1299 and H1299/p53 cell apoptosis, reduced cytosolic cytochrome *c* accumulation (Fig. 3B), and inhibited caspase-3 activation in response to gemcitabine (data not shown), suggesting that gemcitabine-induced apoptosis might mediate, in part, through the downregulation of Bcl-2 protein. Taken together, these results indicate that the

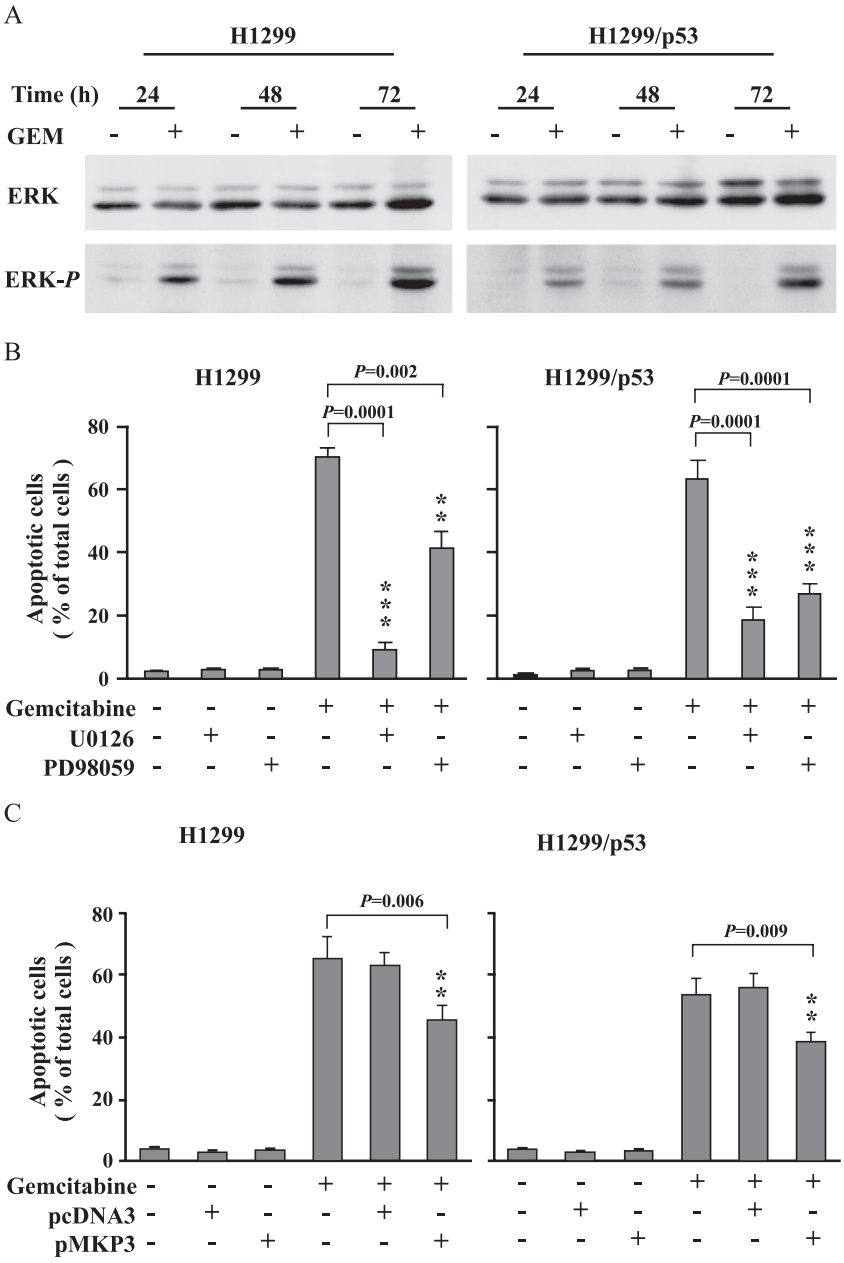

Fig. 4. Requirement of ERK activation in gemcitabine-induced apoptosis. H1299 and H1299/p53 cells were treated with 1 μM gemcitabine for indicated time periods (24, 48, and 72 h). After treatment, (A) total ERK or phosphorylated-ERK was analyzed by Western blot using specific antibodies. (B) U0126- and PD98059-protected cells against gemcitabine-triggered apoptotic cell death. Cells were pretreated with 20 μM U0126 or 40 μM PD98059 for 3 h before adding 1 μM gemcitabine for 3 days. Apoptotic cells were quantified by TUNEL assay using flow cytometry. (C) Ectopic expression of MKP3 inhibited gemcitabine-induced apoptosis. Cells were transfected with 1 μg of pcDNA3 or pMKP3 expression plasmid. After transfection for 24 h, cells were washed and incubated in the presence or absence of gemcitabine for another 48 h. Apoptotic cells were determined by TUNEL assay.

mitochondrial cellular execution pathway plays a critical role in gemcitabine-induced apoptosis in both H1299 and H1299/p53 cells.

3.3. Gemcitabine-induced apoptosis is mediated, in part, through the ERK pathway

The MAPK signaling pathway has been shown to be activated in response to certain chemotherapeutic drugs (Lee and McCubrey, 2002). To investigate whether gemcitabine treatment could lead to MAPK activation, lysates obtained at various times from gemcitabine-treated cells were subjected to Western blot analysis using anti-MAPKs and anti-phospho-MAPKs antibodies to detect phosphorylated (and therefore activated) MAPKs. The protein level of ERK1/2 of H1299 cells treated with gemcitabine did not change compared with control cultures. However, the phospho-ERK1/2 increased in both H1299 and H1299/p53 cells treated with gemcitabine (Fig. 4A). ERK1/2 activation was observed at 24 h after gemcitabine treatment and was sustained up to 72 h. The total protein and phosphorylated JNK and p38 were not altered in gemcitabine-treated cells (data not shown). MAP/ERK kinase (MEK), the kinase lying directly upstream of ERK1/2, are responsible for ERK1/2 activation. Two specific inhibitors of MEK, PD98059 and U0126, have been developed, which are highly selective in their inhibition of the ERK1/2 pathway (English and Cobb, 2002). These two inhibitors were used in our study to evaluate whether ERK1/2 activation is required for gemcitabine-induced apoptosis. Cells were pretreated with various doses of PD98059 or U0126 for 1 h before addition of gemcitabine. Quantitation of phosphorylated ERK1/2 and apoptotic cells demonstrated that 20 μM U0126 could significantly inhibit apoptosis in response to gemcitabine treatment (Fig. 4B). Similar results were also obtained when H1299 cells were treated with PD98059 despite the fact that U0126 exhibited higher protective efficacy than PD98059 (Fig. 4B). Next, we expressed the MKP3 in H1299 and H1299/p53 cells to further determine whether the phosphorylated ERK is a critical event required for gemcitabine-induced cell death. MKP3, a cytosolic phosphatase, has been shown to specifically dephosphorylate phosphor-ERK (Burnet et al., 1999). As shown in Fig. 4C, in the presence of MKP3 expression, gemcitabine-induced apoptosis was significantly decreased. These data suggest that ERK activation is necessary for gemcitabine-induced apoptosis.

3.4. Role of ERK in mediating Bcl-2 downregulation, cytochrome c release, and caspase activation in gemcitabine-treated cells

We next investigated whether Bcl-2 downregulation and cytochrome *c* release in response to gemcitabine treatment were dependent on ERK activation. Cells were treated with 1 μM gemcitabine in the absence or presence of 20 μM U0126, after which cell extracts were prepared and the protein levels of Bcl-2 and cytochrome *c* were measured by Western blotting. Similar to Figs. 2C and 3A, cytosolic cytochrome *c* increased and Bcl-2 decreased in the gemcitabine-treated cells compared with control cultures. These processes were inhibited in the presence of the inhibitor of ERK signaling pathway, U0126 (Fig. 5). These findings suggest that ERK acts upstream of Bcl-2 reduction and cytochrome *c* release to exert its apoptotic influence in gemcitabine-treated H1299 and H1299/p53 cells.

3.5. Akt inactivation correlated with gemcitabine-triggered apoptosis

Growing evidence indicate that inactivation of Akt is closely linked to the cell death of cancer cell to a broad spectrum of apoptotic stimuli (Datta et al., 1999). To determine whether Akt is also inactivated in gemcitabine-induced apoptosis, Akt activation was analyzed by Western blotting using antibodies recognizing the Thr308- and Ser473-phosphorylated form of Akt. Fig. 6A shows that basal Akt phosphorylation was found in H1299 and H1299/p53 cells. However, there was a significant decline in the levels of phosphorylated Akt at 24–72 h of gemcitabine treatment. To assess the role of inactivated Akt in the apoptosis induced by gemcitabine, cell-permeable LY294002, which is a PI3K inhibitor, was used to inhibit the Akt pathway. As shown in Fig. 6B, LY294002 significantly accelerated gemcitabine-mediated apoptosis in H1299 and H1299/p53 cells.

3.6. Induction of NF-kB DNA-binding activity by gemcitabine

It is well documented that NF-kB and AP-1 are the nuclear targets of ERK signaling pathways. We further examined whether AP-1 and/or NF-kB could be activated in

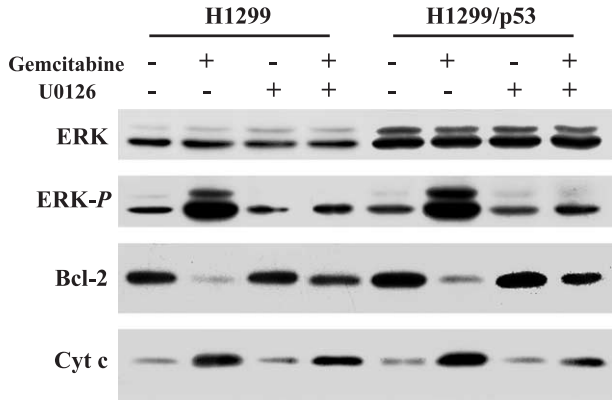

Fig. 5. Bcl-2 and cytochrome *c* as downstream targets of ERK activation in gemcitabine-treated cells. H1299 and H1299/p53 cells were pretreated with 20 μM U0126 for 3 h before adding 1 μM gemcitabine for 2 days. The lysates were prepared and subjected to Western blot analysis for the expression pattern of ERK, phospho-ERK, Bcl-2, and cytochrome *c*.

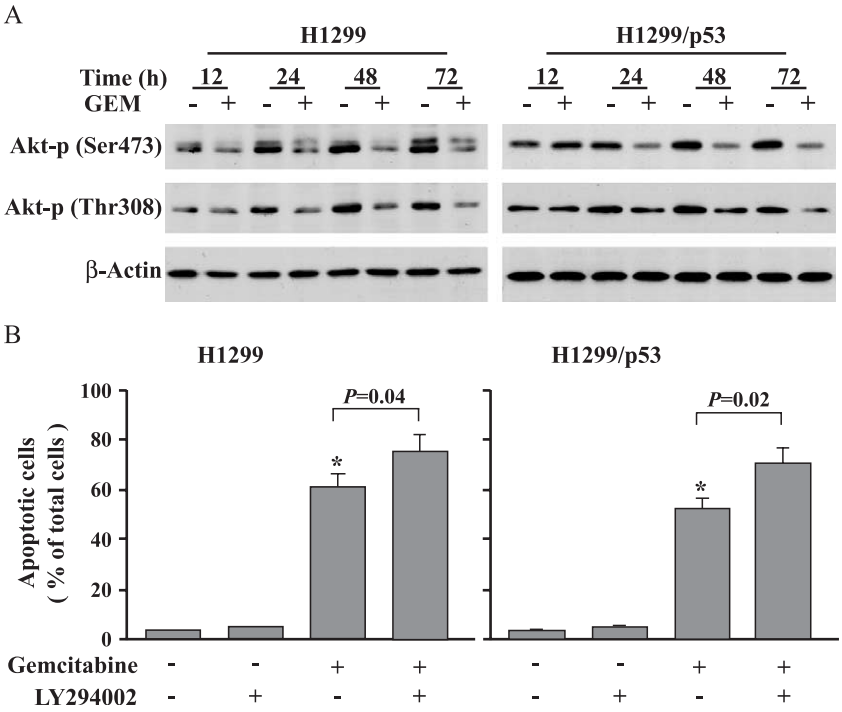

Fig. 6. Inactivation of Akt by gemcitabine. (A) Cells were treated with 1 μM gemcitabine for various time points. The expression levels of phosphor-Akt proteins were analyzed by Western blot using anti-phospho-Akt antibodies. (B) Promotion of gemcitabine-induced apoptosis by LY294002. Cells were treated with gemcitabine in the presence or absence of LY294002 for 48 h. After treatment, apoptotic cells were measured by TUNEL assay.

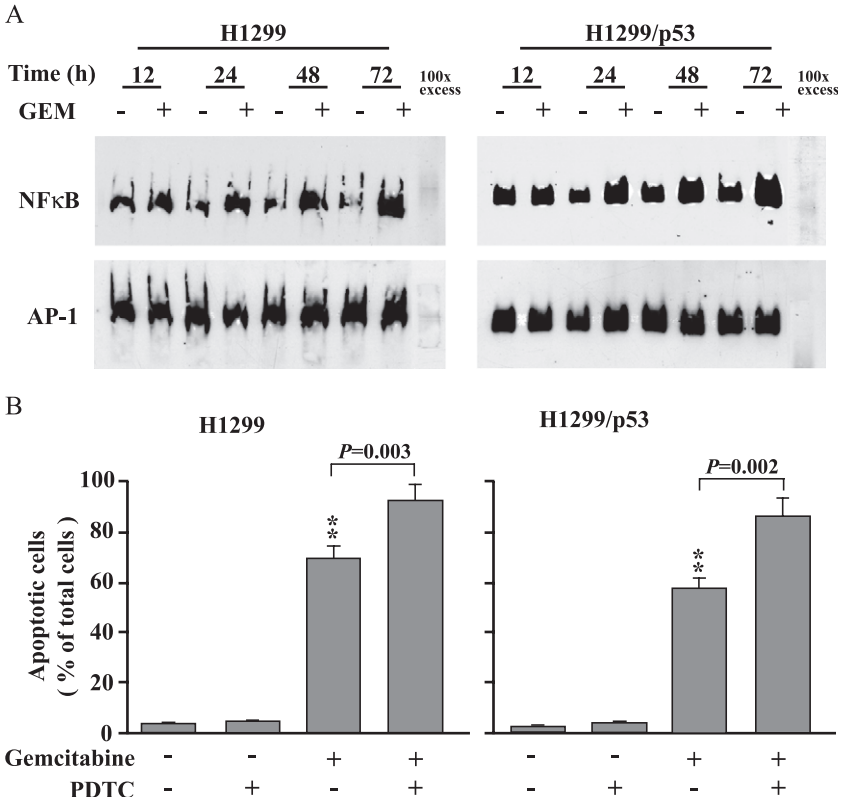

Fig. 7. Enhancement of NF-kB DNA-binding activity by gemcitabine. (A) DNA-binding activity. Cells were treated with 1 μM gemcitabine for indicated time periods. Nuclear extracts were prepared, and electrophoretic mobility shift assay was performed using biotin-labeled NF-kB and AP-1 consensus-binding sequence. (B) Inhibition of NF-kB signaling accelerated gemcitabine-induced apoptosis. Cells were treated with or without 1 μM gemcitabine in the presence or absence of various concentrations of PDTC for 48 h. Apoptotic cells were determined by TUNEL assay.

gemcitabine-treated H1299 and H1299/p53 cells; the DNA-binding activity of AP-1 and NF-kB in nuclear extracts was analyzed by electrophoretic gel mobility shift assay using biotin-labeled oligonucleotides, which contained the consensus-binding motif of AP-1 or NF-kB. As depicted in Fig. 7A, DNA-binding activity of NF-kB was persistently increased upon gemcitabine treatment. This binding is specific since it could be blocked by preincubation with a 100-fold excess amount of unlabeled NF-kB response oligonucleotides. Densitometric analysis showed that the binding activity of NF-kB at 24 h gemcitabine treatment was increased to 3- and 2.5-fold vs. controls in H1299 and H1299/p53 cells, respectively. The NF-kB DNA-binding activity remained enhanced for 48–72 h after gemcitabine treatment. In contrast, the DNA-binding activity of AP-1 was not affected upon gemcitabine treatment (Fig. 7A).

To determine the effect of inhibition of NF-kB activation on gemcitabine-triggered apoptosis, we examined the impact of a NF-kB inhibitor, PDTC (Wright et al., 2002). As depicted in Fig. 7B, treatment with PDTC alone did not alter the incidence of apoptosis. However, PDTC treatment sensitized cells to gemcitabine. The fraction of apoptotic cells increased significantly in the combined PDTC/gemcitabine treatment compared with the gemcitabine treatment alone.

4. Discussion

The ability of cytotoxic agents to limit tumor growth may be due to their capacity to inhibit cancer cell proliferation or to increase cancer cell death. Gemcitabine is a promising drug for treatment of lung cancer (Bunn and Kelly, 1998). However, the cell-killing mechanism of gemcitabine in NSCLC is still not well characterized, and there have been no reports on the correlation between growth inhibition, cell cycle, and apoptosis. Since p53 influences cell cycle progression and apoptotic response, mutation of the *p53* gene is the most commonly identified genetic abnormality in lung cancer. Thus, we investigated the effect of gemcitabine on a large cell lung cancer H1299 cell line (which is a p53-null cell line) (Nguyen et al., 1996) while altering the p53 status by ectopic expression of wild-type *p53* gene and allowing a direct comparison between cytotoxicity of this drug in the presence or absence of functional p53 protein (Fig. 8). We found that treatment of H1299 cells with gemcitabine resulted in S-phase arrest and treatment of H1299/p53 cells resulted in G1- and S-phase accumulation, with constitutively expressed p53 only detected in H1299/p53 cells, indicating a role for p53 in controlling G1 checkpoints. In our study, the $p21^{CIP1/WAF1}$ protein was markedly induced during gemcitabine treatment of the H1299/p53 cells but slightly induced in H1299 cells (Fig. 1C), providing a possible explanation for the observed G1 accumulation. However, the induction of p53 by gemcitabine in H1299/p53 cells could not be detected. This

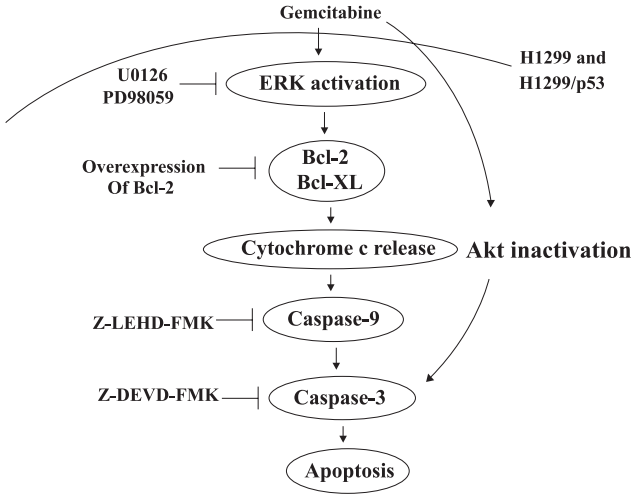

Fig. 8. The schematic representation of gemcitabine-induced p53-independent apoptotic pathway in human NSCLC H1299 and H1299/p53 cell lines.

result was unexpected because $p21^{CIP1/WAF1}$ is transcriptionally regulated by p53 (Bargonetti and Manfredi, 2002) and is specifically induced in p53-dependent cellular responses to DNA damage (Bargonetti and Manfredi, 2002). Several studies have reported the existence of p53-independent G1 arrest and $p21^{CIP1/WAF1}$ induction (Strasser et al., 1994; Ko and Prives, 1996; Loignon et al., 1997). This pathway seems to be selectively activated by different chemotherapeutic agents, depending on the cellular context. Details about this issue remain to be further explored.

Loss of p53 is linked to resistance to chemotherapy in some tumor cells and to increased sensitivity in others (Chang et al., 2000; Pirollo et al., 2000). Several studies have demonstrated that reintroduction of wild-type p53 into tumor cells has led to a variety of effects, including growth arrest and apoptosis (Gomez-Manzano et al., 1996; Chang et al., 2000; Pirollo et al., 2000). But the results obtained for most of tumor cell types are controversial. In the study by Galmarini et al. (2002), cancer cells carrying a wild-type p53 are more sensitive to gemcitabine than cells with a mut-p53, which were also observed by others (Chen et al., 2000; Feng et al., 2000). Inactivation of p53 by transfection of p53 normal colon cancer cells with the human papillomavirus *E6* gene resulted in decreased gemcitabine-mediated cytotoxicity and apoptosis than p53 normal cells (Chen et al., 2000). Conversely, it has been reported that p53 function did not impact gemcitabine cytotoxicity in human bladder cancer cells (Kielb et al., 2001; Fechner et al., 2003) and in NSCLC cell lines (Edelman et al., 2001). In the present study, there was a definitely higher tendency of the null-p53 H1299 cells to undergo apoptosis as compared with the wild-type p53 cells (H1299/p53 cells). However, H1299/p53 cells also retained the capability to die by apoptosis; this process must probably be executed by an alternative p53-independent pathway. Our data suggest that whereas p53 is an important factor influencing the cell cycle distribution in

response to gemcitabine treatment, wild-type p53 function is not an absolute requirement for gemcitabine-induced cytotoxicity; apoptosis of cells with both functional and nonfunctional p53 were observed and attributed to the activation of a p53-independent pathway. In the study by Cascallo et al. (2000), viral transfection of p53 first with p53 overexpression severely compromised treatment with gemcitabine. If p53 is reintroduced into cells that have already been treated with gemcitabine, p53 recognizes DNA damage and commits to apoptosis. Under different schedules of administration, exogenous p53 could lead to different results. The introduction of wild-type *p53* gene via adenovirus, under appropriate sequence of administration, will be a very promising approach to improve the efficiency of chemotherapeutic agents. These results demonstrated that the interaction between p53 function and gemcitabine activity is not a predictable event, and may be dependent on cellular context and experimental differences.

Proteins including the MAPK family are activated through a specific phosphorylation cascade, and constitute important mediators of signal transduction processes that serve to coordinate the cellular response to a variety of extracellular stimuli (Chen et al., 2001). The ERK pathway plays a major role in regulating cell growth and differentiation, which is highly induced in response to growth factors, cytokines, and phorbol esters (Aikawa et al., 1997). Many studies have concluded the general view that activation of the ERK pathway drives a survival signal that counteracts proapoptotic effects, especially with JNK, p38, and two MAPK subfamilies (Xia et al., 1995; Graves et al., 1996; Chen et al., 1996; Brenner et al., 1997). It has been reported that an inhibition of ERK signaling leads to increased sensitivity of ovarian cancer cell lines to cisplatin (*cis*-diamminedichloroplatinum; CDDP) (Hayakawa et al., 1999; Persons et al., 1999). However, in the present study, we have provided evidence that activation of ERK is important for the induction of gemcitabine-induced apoptosis in both H1299 and H1299/p53 cells. Gemcitabine treatment resulted in a high and sustained activation of ERK in these cells. Similarly, the increased level of ERK activity was observed on gemcitabine-induced apoptosis on breast MDA-MB-453 cells (Nelson and Fry, 2001). Accumulating evidence showed that, in different cell types such as HeLa cells, osteosarcoma cells, neuroblastoma cells, and mouse embryonic fibroblasts, activation of ERK by means of different agents such as cisplatin, paclitaxel, adriamycin, and etoposide is involved in the induction of apoptosis and cell cycle arrest (Wang et al., 2000; Bacus et al., 2001; Tang et al., 2002). Survival was increased in tumor cells through the inhibition of signaling through ERK by the MEK1 inhibitor PD98059 or U0126 in these studies and also in our study. Our result also showed that ectopic expression of MKP3, a specific phosphor-ERK phosphatase (Burnet et al., 1999), markedly decreased gemcitabine-mediated ERK phosphorylation and apoptotic cell death (Fig. 4C). It has been reported that gemcitabine caused a considerable percentage of the cell population to commit to apoptosis, and the major effect on signaling pathways was an accompanying elevation of activated p38 in MDA-MB-453 (Nelson and Fry, 2001) and in human pancreatic cancer cells (Habiro et al., 2004). However, the activation of p38 and JNK could not be detected in gemcitabine-treated H1299 and H1299/p53 cells in the present study. Such differential effects observed from one study to another could reflect cell type or treatment specificity. Recently, it has been shown that ERK phosphorylates and stabilizes p53 and alters p53 target gene expression after cisplatin treatment, but the biological consequences have not yet been studied (Persons et al., 2000; DeHaan et al., 2001). It has been suggested that ERK may mediate cisplatin-induced accumulation of p53 to trigger apoptosis (Park et al., 2001). In our study, the proapoptotic effect of gemcitabine-induced activation of ERK is independent of the p53 status of the cells.

Growing evidence indicated that NF-kB is a nuclear target of ERK signaling pathways. NF-kB is a ubiquitously expressed transcription factor that is involved in a wide spectrum of cellular functions like cell cycle control, stress adaptation, inflammation, and control of apoptosis (Bours et al., 2000). Several studies have indicated that chemotherapeutic agents, such as etoposide, CPT-11, adriamycin, vincristine, and taxol, can induce NF-kB activation (Das and White, 1997; Wang et al., 1999a,b). Once activated by chemotherapy, NF-kB has been shown to provide an antiapoptotic function by promoting cell survival of colorectal carcinoma and NSCLC cell lines (Wang et al., 1999a,b; Jones et al., 2000). The present study showed that gemcitabine induced NF-kB activation in NSCLC H1299 cells in a p53-independent manner. This observed gemcitabine-induced NF-kB activation is supported by other studies (Jones et al., 2000; Arlt et al., 2003). Furthermore, treatment with the NF-kB inhibitor, PDTC, significantly enhanced gemcitabine-induced cell death. These finding suggests that NF-kB activation provides a cell survival signal following gemcitabine treatment. Other studies have confirmed that inhibition of NF-kB would sensitize tumor cells to undergo gemcitabine-induced apoptosis (Jones et al., 2000). Previous reports demonstrate that NF-kB may contribute to antiapoptotic survival responses by transcriptional regulation of cIAPs and Bcl-2 homologue A1 (Wang et al., 1998, 1999a,b). In contrast, NF-kB is also considered a proapoptotic factor because of its involvement in the expression of some apoptotic molecules, including Fas and FasL (Kasibhatla et al., 1998; Ashkenazi and Dixit, 1999). The reason for these controversial findings in the role of NF-kB activation in apoptosis remains to be determined, but may relate to differences in the biological differences between the investigated cell types and/or different stimuli. However, it seems to be the integration of some additional signals in combination with NF-kB activation that ultimately determines the role of NF-kB in proapoptotic or antiapoptotic responses to extracellular stimuli.

The serine/threonine protein kinase Akt is increasingly recognized as a key cellular signal that promotes cell proliferation and survival. Akt activation has been investigated in NSCLCs, whereas the downregulation of Akt has been found for taxol and MAPK inhibitor-induced apoptosis (MacKeigan et al., 2002). Our results indicate that gemcitabine leading to tumor cell apoptosis is attributable to the inactivation of the PI3K–Akt pathway because the level of phosphorylated Akt was gradually and persistently reduced after exposure to gemcitabine. Importantly, our study provides strong evidence to support the conclusion that the enhanced apoptosis observed with a combination of gemcitabine and PI3K inhibitor (LY294002) is associated with a reduction of the activated Akt. It has been reported that Akt plays a role in gemcitabine resistance of the pancreatic adenocarcinoma cell lines (Ng et al., 2000). Conversely, a recent report showed that gemcitabine did not affect the Akt phosphorylation in pancreatic carcinoma cell lines; furthermore, treatment with the specific PI3K/Akt inhibitor, LY294002, did not enhance gemcitabine-induced cell death (Arlt et al., 2003). These discrepant findings may be attributed to the differences of cellular context and/or stimuli.

Two major apoptosis pathways have been described from mammalian cells. One is receptor-related and involves caspase-8, such as Fas/APO-1-associated death domain protein to death receptors on extracellular ligand binding (Cryns and Yuan, 1998; Muzio et al., 1998). The other pathway is associated with mitochondria and involves cytochrome *c* release-dependent activation of caspase-9 through Apaf-1 (Zou et al., 1997, 1999). The Bcl-2 family of proteins has been identified as a key regulator of apoptosis in many cellular systems. This family is common divided into both antiapoptotic (Bcl-2, Bcl-X_L, Mcl-1, and A1) and proapoptotic members (Bax, Bak, Bad, Bid, Bik, and Bcl-Xs). It had been reported that cellular Bcl-2 content was directly correlated with the cytotoxicity of gemcitabine in pancreatic carcinoma (Bold et al., 1999). Bcl-X_L antisense oligonucleotide treatment increases the sensitivity of pancreatic cancer cells to gemcitabine (Xu et al., 2001). In the current study, downregulation of Bcl-2 and Bcl-X_L increased levels of cytosolic cytochrome *c*, and activation of caspase-9 in gemcitabine-treated cells is observed. Overexpression of Bcl-2 protein by adeno-Bcl-2 viral vector infection markedly blocked gemcitabine-mediated apoptotic cell death in both H1299 and H1299/p53 cells, suggesting that Bcl-2 family-dependent mitochondrial pathway plays a role in mediating gemcitabine-induced apoptosis. The ability of the U0126 to reduce this effect implies that the ERK signaling pathway functions upstream of Bcl-2 downregulation and cytochrome *c* release in the induction of cell death upon gemcitabine treatment. Our findings may indeed be true, but differential effects observed from one study to another could reflect cell type specificity and need caution with respect to the generality of this pathway.

In conclusion, the current study showed that gemcitabine exerts its antitumor effect by the activation of the apoptotic machinery in NSCLC H1299 cells at clinically relevant concentrations around 1 μM. Although apoptosis could more easily be induced in the H1299 (null-p53 cells), the process was also inducible in the H1299/p53 (wild-type p53 cells), indicating the presence of alternative p53-independent pathways. Our observations suggest that the p53-independent ERK activation plays an important role in mediating gemcitabine-induced apoptosis of H1299 and H1299/p53 cells, and functions upstream of reduction of the Bcl-2 protein and accumulation of cytosolic cytochrome *c* to initiate the apoptotic signal (Fig. 8). Furthermore, inactivation of Akt was also attributed to gemcitabine-induced apoptosis.

Acknowledgments

This work was supported by Taichung Veterans General Hospital grants TCVGH-937318D and TCVGH-934705D, and National Science Council grant NSC92-2311-B-075A-001.

References

Achanta, G., Pelicano, H., Feng, L., Plunkett, W., Huang, P., 2001. Interaction of p53 and DNA-PK in response to nucleoside analogues: potential role as a sensor complex for DNA damage. Cancer Res. 61, 8723–8729.

Aikawa, R., Komuro, I., Yamazaki, T., Zou, Y., Kudoh, S., Tanaka, M., Shiojima, I., Hiroi, Y., Yazaki, Y., 1997. Oxidative stress activates extracellular signal-regulated kinases through Src and Ras in cultured cardiac myocytes of neonatal rats. J. Clin. Invest. 100, 1813–1821.

Arlt, A., Gehrz, A., Muerkosteeer, S., Vorndamm, J., Kruse, M.-L., Folsch, U.R., Schafer, H., 2003. Role of NF-kB and Akt/PI3K in the resistance of pancreatic carcinoma cell lines against gemcitabine-induced cell death. Oncogene 22, 3243–3251.

Ashkenazi, A., Dixit, V.M., 1999. Apoptosis control by death and decoy receptors. Curr. Opin. Cell Biol. 11, 255–260.

Bacus, S.S., Gudkov, A.V., Lowe, M., Lyass, L., Yung, Y., Komarov, A.P., Keyomarsi, K., Yarden, Y., Seger, R., 2001. Taxol-induced apoptosis depends on MAP kinase pathways (ERK and p38) and is independent of p53. Oncogene 20, 147–155.

Bargonetti, J., Manfredi, J.J., 2002. Multiple roles of the tumor suppressor p53. Curr. Opin. Oncol. 14, 86–91.

Berger, R., Duncan, W.R., Martinez, C.A., 1993. Nonradioactive gel mobility shift assay using chemiluminescent detection. BioTechniques 15, 650–652.

Bold, R.J., Chandra, J., McConkey, D.J., 1999. Gemcitabine-induced programmed cell death (apoptosis) of human pancreatic carcinoma is determined by Bcl-2 content. Ann. Surg. Oncol. 6, 279–285.

Bours, V., Bentires-Alj, M., Hellin, A.-C., Viatour, P., Robe, P., Delhalle, S., Benoit, V., Merville, M.-P., 2000. Nuclear factor-kB, cancer, and apoptosis. Biochem. Pharmacol. 60, 1085–1090.

Brenner, B., Koppenhoefer, U., Weinstock, C., Linderkamp, O., Lang, F., Gulbins, E., 1997. Fas- or ceramide-induced apoptosis is mediated by a Rac1-regulated activation of Jun N-terminal kinase/p38 kinases and GADD153. J. Biol. Chem. 272, 22173–22181.

Bunn Jr., P.A., Kelly, K., 1998. New chemotherapeutic agents prolong survival and improve quality of life in non-small cell lung cancer: a review of the literature and future directions. Clin. Cancer Res. 4, 1087–1100.

Burnet, A., Roux, D., Lenormand, P., Dowd, S., Keyse, S., Pouyssegur, J., 1999. Nuclear translocation of p42/p44 mitogen-activated protein kinase is required for growth factor-induced gene expression and cell cycle entry. EMBO J. 18, 664–674.

Cascallo, M., Calbo, J., Gelpi, J.L., Mazo, A., 2000. Modulation of drug cytotoxicity by reintroduction of wild-type *p53* gene (Ad5CMV-p53) in human pancreatic cancer. Cancer Gene Ther. 7, 545–556.

Chang, E.H., Pirollo, K.F., Bouker, K.B., 2000. Tp53 gene therapy: a key to modulating resistance to anticancer therapies? Mol. Med. 6, 358–364.

Chen, Y.R., Wang, X., Templeton, D., Davis, R.J., Tan, T.H., 1996. The role of c-Jun N-terminal kinase (JNK) in apoptosis induced by ultraviolet C and gamma radiation. Duration of JNK activation may determine cell death and proliferation. J. Biol. Chem. 271, 31926–31929.

Chen, M., Hough, A.M., Lawrence, T.S., 2000. The role of p53 in gemcitabine-mediated cytotoxicity and radiosensitization. Cancer Chemother. Pharmacol. 45, 369–374.

Chen, Z., Gibson, T.B., Robinson, F., Silvestro, L., Pearson, G., Xu, B., Wright, A., Vanderbilt, C., Cobb, M.H., 2001. MAP kinases. Chem. Rev. 101, 2449–2476.

Chiba, I., Takahashi, T., Nau, M.M., D'Amico, D., Curiel, D.T., Mitsudomi, T., Buchhagen, D.L., Carbone, D., Piantadosi, S., Koga, H., 1990. Mutations in the *p53* gene are frequent in primary, resected non-small cell lung cancer. Oncogene 5, 1603–1610.

Cohen, G.M., 1997. Caspases: the executioners of apoptosis. Biochem. J. 326, 1–16.

Cryns, V., Yuan, J., 1998. Proteases to die for. Genes Dev. 12, 1551–1570.

Das, K.C., White, C.W., 1997. Activation of NF-kB by antineoplastic agents: role of protein kinase C. J. Biol. Chem. 272, 14914–14920.

Datta, S.R., Brunet, A., Greenberg, M.E., 1999. Cellular survival: a play in three Akts. Genes Dev. 13, 2905–2927.

DeHaan, R.D., Yazlovitskaya, E.M., Persons, D.L., 2001. Regulation of p53 target gene expression by cisplatin-induced extracellular signal-regulated kinase. Cancer Chemother. Pharmacol. 48, 383–388.

Edelman, M.J., Quam, H., Mullins, B., 2001. Interactions of gemcitabine, carboplatin and paclitaxel in molecularly defined non-small-cell lung cancer cell lines. Cancer Chemother. Pharmacol. 48, 141–144.

English, J.M., Cobb, M.H., 2002. Pharmacological inhibitors of MAPK pathways. Trends Pharmacol. Sci. 23, 40–45.

Fechner, G., Perabo, F.G.E., Schmidt, D.H., Haase, L., Ludwig, E., Schueller, H., Blatter, J., Muller, S.C., Albers, P., 2003. Preclinical evaluation of a rediosensitizing effect of gemcitabine in *p53* mutant and p53 wild type bladder cancer cells. Urology 61, 468–473.

Feng, L., Achanta, G., Pelicano, H., Zhang, W., Plunkett, W., Huang, P., 2000. Role of p53 in cellular response to anticancer nucleoside analog-induced DNA damage. Int. J. Mol. Med. 5, 597–604.

Galmarini, C.M., Clarke, M.L., Falette, N., Puisieux, A., Mackey, J.R., Dumontet, C., 2002. Expression of a non-functional p53 affects the sensitivity of cancer cells to gemcitabine. Int. J. Cancer 97, 439–445.

Gomez-Manzano, C., Fueyo, J., Kyritsis, A.P., Steck, P.A., Roth, J.A., McDonnell, T.J., Steck, K.D., Levin, V.A., Yung, W.K., 1996. Adenovirus-mediated transfer of the *p53* gene produces rapid and generalized death of human glioma cells via apoptosis. Cancer Res. 56, 694–699.

Graham, K.A., Leithoff, J., Coe, I.R., Mowles, D., Mackey, J.R., Young, J.D., Cass, C.E., 2000. Differential transport of cytosine-containing nucleosides by recombinant human concentrative nucleoside transporter protein hCNT1. Nucleosides Nucleotides Nucleic Acids 19, 415–434.

Graves, J.D., Draves, K.E., Craxton, A., Saklatvala, J., Krebs, E.G., Clark, E.A., 1996. Involvement of stress-activated protein kinase and p38 mitogen-activated protein kinase in mIgM-induced apoptosis of human B lymphocytes. Proc. Natl. Acad. Sci. U. S. A. 93, 13814–13818.

Habiro, A., Tanno, S., Koizumi, K., Izawa, T., Nakano, Y., Osanai, M., Mizukami, Y., Okumura, T., Kohgo, Y., 2004. Involvement of p38 mitogen-activated protein kinase in gemcitabine-induced apoptosis in human pancreatic cancer cells. Biochem. Biophys. Res. Commun. 316, 71–77.

Hayakawa, J., Ohmichi, M., Kurachi, H., Ikegami, H., Kimura, A., Matsuoka, T., Jikihara, H., Mercola, D., Murata, Y., 1999. Inhibition of extracellular signal-regulated protein kinase or c-Jun N-terminal protein kinase cascade, differentially activated by cisplatin, sensitizes human ovarian cancer cell line. J. Biol. Chem. 274, 31648–31654.

Hollstein, M., Sidranski, D., Vogelstein, B., Harris, C.C., 1991. *p53* mutations in human cancers. Science 253, 49–53.

Jones, D.R., Broad, R.M., Madrid, L.V., Baldwin Jr., A.S., Mayo, M.W., 2000. Inhibition of NF-kB sensitizes non-small-cell lung cancer cells to chemotherapy-induced apoptosis. Ann. Thorac. Surg. 70, 930–937.

Joza, N., Kroemer, G., Penninger, J.M., 2002. Genetic analysis of the mammalian cell death machinery. TRENDS Genet. 18, 142–149.

Kasibhatla, S., Brunner, Y., Genestier, L., Echeverri, F., Mahboubi, A., Green, D.R., 1998. DNA damaging agents induce expression of Fas ligand and subsequent apoptosis in T lymphocytes via the activation of NF-kappa B and AP-1. Mol. Cell 1, 543–551.

Kielb, S.J., Shah, N.L., Rubin, M.A., Sanda, M.G., 2001. Functional *p53* mutation as a molecular determinant of paclitaxel and gemcitabine susceptibility in human bladder cancer. J. Urol. 166, 482–487.

Ko, L.J., Prives, C., 1996. P53: puzzle and paradigm. Genes Dev. 10, 1054–1072.

Lee Jr., J.T., McCubrey, J.A., 2002. The Raf/MEK/ERK signal transduction cascade as a target for chemotherapeutic intervention in leukemia. Leukemia 16, 486–507.

Loignon, M., Fetni, R., Gordon, A.J., Drobetsky, E.A., 1997. A p53-independent pathway for induction of p21waf1cip1 and concomitant G1 arrest in UV-irradiated human skin fibroblasts. Cancer Res. 57, 3390–3394.

MacKeigan, J.P., Taxman, D.J., Hunter, D., Earp II, H.S., Graves, L.M., Ting, J.P.-Y., 2002. Inactivation of the antiapoptotic phosphatidylinositol 3-kinase–Akt pathway by the combined treatment of taxol and mitogen-activated protein kinase inhibition. Clin. Cancer Res. 8, 2091–2099.

Mackey, J.R., Mani, R.S., Selner, M., Mowles, D., Young, J.D., Belt, J.A., Crawford, C.R., Cass, C.E., 1998. Functional nucleoside transporters are required for gemcitabine influx and manifestation of toxicity in cancer cell lines. Cancer Res. 58, 4349–4357.

Muzio, M., Stockwell, B.R., Stennicke, H.R., Salvesen, G.S., Dixit, V.M., 1998. An induced proximity model for caspase-8 activation. J. Biol. Chem. 273, 2926–2930.

Nabhan, C., Krett, N., Gandhi, V., Rosen, S., 2001. Gemcitabine in hematologic malignancies. Curr. Opin. Oncol. 13, 14–521.

Nelson, J.M., Fry, D.W., 2001. Akt, MAPK (Erk1/2), and p38 act in concert to promote apoptosis in response to ErbB receptor family inhibition. J. Biol. Chem. 276, 14842–14847.

Ng, S.S.W., Tsao, M.S., Chow, S., Hedley, D.W., 2000. Inhibition of phosphatidylinositide 3-kinase enhances gemcitabine-induced apoptosis in human pancreatic cancer cells. Cancer Res. 60, 5451–5455.

Nguyen, D.M., Spitz, F.R., Yen, N., Cristiano, R.J., Roth, J.A., 1996. Gene therapy for lung cancer: enhancement of tumor suppression by a combination of sequential systemic cisplatin and adenovirus-mediated *p53* gene transfer. J. Thorac. Cardiovasc. Surg. 112, 1372–1376.

Pace, E., Melis, M., Siena, L., Bucchieri, F., Vignola, A.M., Profita, M., Gjomarkaj, M., Bonsignore, G., 2000. Effects of gemcitabine on cell proliferation and apoptosis in non-small-cell lung cancer (NSCLC) cell lines. Cancer Chemother. Pharmacol. 46, 467–476.

Park, S.A., Park, H.J., Lee, B.I., Ahn, Y.H., Kim, S.U., Choi, K.S., 2001. Bcl-2 blocks cisplatin-induced apoptosis by suppression of ERK-mediated p53 accumulation in B104 cells. Brain Res. Mol. Brain Res. 93, 18–26.

Persons, D.L., Yazlovitskaya, E.M., Cui, W., Pelling, J.C., 1999. Cisplatin-induced activation of mitogen-activated protein kinases in ovarian carcinoma cells: inhibition of extracellular signal-regulated kinase activity increases sensitivity to cisplatin. Clin. Cancer Res. 5, 1007–1014.

Persons, D.L., Yazlovitskaya, E.M., Pelling, J.C., 2000. Effect of extracellular signal-regulated kinase on p53 accumulation in response to cisplatin. J. Biol. Chem. 275, 35778–35785.

Pirollo, K.F., Bouker, K.B., Chang, E.H., 2000. Does p53 status influence tumor response to anticancer therapies? Anticancer Drugs 11, 419–432.

Rodgers, J.T., Patel, P., Hennes, J.L., Bolognia, S.L., Mascotti, D.P., 2000. Use of biotin-labeled nucleic acids for protein purification and agarose-based chemiluminescent electromobility shift assays. Anal. Biochem. 277, 254–259.

Shelton, J.G., Steelman, L.S., Lee, J.T., Knapp, S.L., Blalock, W.L., Moye, P.W., Franklin, R.A., Pohnert, S.C., Mirza, A.M., McMahon, M., McCubrey, J.A., 2003. Effects of the RAF/MEK/ERK and PI3K/AKT signal transduction pathways on the abrogation of cytokine-dependence and prevention of apoptosis in hematopoietic cells. Oncogene 22, 2478–2492.

Stambolic, V., Mak, T.W., Woodgett, J.R., 1999. Modulation of cellular apoptotic potential: contributions to oncogenesis. Oncogene 18, 6094–6103.

Strasser, A., Harris, A.W., Jacks, T., Cory, S., 1994. DNA damage can induce apoptosis in proliferating lymphoid cells via p53-independent mechanisms inhibitable by Bcl-2. Cell 79, 329–339.

Tang, D., Wu, D., Hirao, A., Lahti, J.M., Liu, L., Mazza, B., Kidd, V.J., Mak, T.W., Ingram, A.J., 2002. ERK activation mediates cell cycle arrest and apoptosis after DNA damage independently of p53. J. Biol. Chem. 277, 12710–12717.

Wang, C.Y., Mayo, M.W., Korneluk, R.G., Goeddel, D.V., Baldwin, A.S., 1998. NF-kB antiapoptosis: induction of TRAF1 and TRAF2 and cIAP1 and cIAP2 to suppress caspase-8 activation. Science 281, 1680–1683.

Wang, C.Y., Cusack Jr., J.C., Liu, R., Baldwin Jr., A.S., 1999a. Control of inducible chemoresistance: enhanced anti-tumor therapy through increased apoptosis by inhibition of NF-kB. Nat. Med. 5, 412–417.

Wang, C.Y., Guttridge, D.C., Mayo, M.W., Baldwin Jr., A.S., 1999b. NF-kB induces expression of the Bcl-2 homologue A1/Bfl-1 to preferentially suppress chemotherapy-induced apoptosis. Mol. Cell. Biol. 19, 5923–5929.

Wang, X., Martindale, J.L., Holbrook, N.J., 2000. Requirement for ERK activation in cisplatin-induced apoptosis. J. Biol. Chem. 275, 39435–39443.

Woessmann, W., Chen, X., Borkhardt, A., 2002. Ras-mediated activation of ERK by cisplatin induces cell death independently of p53 in osteosarcoma and neuroblastoma cell lines. Cancer Chemother. Pharmacol. 50, 397–404.

Wright, G., Singh, I.S., Hasday, J.D., Farrance, I.K., Hall, G., Cross, A.S., 2002. Endotoxin stress-response in cardiomyocytes: NF-kappaB activation and tumor necrosis factor-alpha expression. Am. J. Physiol, Heart Circ. Physiol. 282, 827–829.

Xia, Z., Dickens, M., Raingeaud, J., Davis, R.J., Greenberg, M.E., 1995. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science 270, 1326–1331.

Xu, Z., Friess, H., Solioz, M., Aebi, S., Kore, M., Kleeff, J., Buchler, M.W., 2001. Bcl-x_L antisense oligonucleotides induce apoptosis and increase sensitivity of pancreatic cancer cells to gemcitabine. Int. J. Cancer 94, 268–274.

Zou, H., Henzel, W.J., Liu, X., Lutschg, A., Wang, X., 1997. Apaf-1, a human protein homologous to *C. elegans* CED-4, participates in cytochrome *c*-dependent activation of caspase-3. Cell 90, 405–413.

Zou, H., Li, Y., Liu, X., Wang, X., 1999. An APAF-1–cytochrome *c* multimeric complex is a functional apoptosome that activates procaspase-9. J. Biol. Chem. 274, 11549–11556.