Cancer Chemother Pharmacol (2002) 50: 397–404
DOI 10.1007/s00280-002-0502-y

ORIGINAL ARTICLE

Willi Woessmann · Xinbin Chen · Arndt Borkhardt

Ras-mediated activation of ERK by cisplatin induces cell death independently of p53 in osteosarcoma and neuroblastoma cell lines

Received: 3 April 2002 / Accepted: 8 July 2002 / Published online: 14 August 2002

Abstract Activation of the mitogen-activated protein kinases ERK1/2 by the chemotherapeutic agent cisplatin has been shown to result in either survival or cell death. The downstream mediators of these opposing effects are unknown, as are the upstream signaling molecules. Activation of ERK is required for accumulation and phosphorylation of p53 following cisplatin treatment. We studied the role of ERK activation after cisplatin treatment under p53-negative and p53-positive conditions using a tetracycline-dependent expression vector in Saos-2 osteosarcoma cells. Dose-dependent activation of ERK first occurred 3–6 h after a 2-h cisplatin incubation and declined after 12–24 h in several tumor cell lines. Incubation of cell lines with the MEK1 inhibitors PD98059 or UO126 after, but not during, cisplatin treatment completely inhibited cisplatin-induced activation of ERK. The activation of ERK by cisplatin was inhibited by transient transfection with dominant-negative Ras-N17 in Saos-2 cells. Treatment of cells with PD98059 or UO126 after cisplatin incubation or inhibition of signaling through ERK by tetracycline-regulated expression of dominant-inhibitory ERK enhanced resistance to cisplatin in p53-negative osteosarcoma cells and reduced cisplatin-induced apoptosis. P53 was stabilized and phosphorylated in a MEK1-dependent manner after cisplatin incubation in Kelly neuroblastoma cells. Inhibition of signaling through ERK increased cell survival after cisplatin treatment in these cells as well. Expression of functional p53 did not change the proapoptotic effects of ERK activation in response to cisplatin in Saos-2 cells. Our results suggest that cisplatin-induced activation of ERK is mediated by Ras. ERK activation increased cisplatin-induced cell death independently of p53 in osteosarcoma and neuroblastoma cell lines.

Keywords Cisplatin · ERK · p53 · Ras · Cell death · Cytotoxicity

W. Woessmann (✉) · A. Borkhardt
Department of Pediatric Hematology and Oncology,
Justus-Liebig-University, 35385 Giessen, Germany
E-mail: wilhelm.woessmann@paediat.med.uni-giessen.de
Tel.: +49-641-9943400
Fax: +49-641-9943429

X. Chen
IMMAG, Medical College of Georgia,
1120 15th Street, Augusta, GA 30912, USA

Introduction

Cisplatin (cis-platinum(II)diaminedichloride) is a chemotherapeutic agent used in the treatment of a wide range of human malignancies, e.g. Ewing sarcoma, osteosarcoma and neuroblastoma. Cellular drug resistance together with a narrow therapeutic range is the major cause of treatment failure [13]. One important mechanism leading to acquired drug resistance is through altered signal transduction to growth or apoptosis [8, 16, 18, 22]. Understanding the detailed signaling events underlying cisplatin-induced apoptosis or repair would help to develop new treatment strategies to improve the therapeutic benefits.

The signaling events implicated in survival, growth arrest or programmed cell death in response to DNA-damaging stress include the activation of mitogen activated protein kinase pathways. Cisplatin has been shown to cause activation of ERK (extracellular regulated kinase, mitogen-activated protein kinase) in ovarian tumor cell lines, neuroblastoma and HeLa cells but not in keratinocytes [5, 15, 21, 23, 25, 30]. The induction of ERK activation in ovarian tumor cells occurs either early or after several hours during continuous treatment with cisplatin [5, 15, 23]. Whether cisplatin activates the ERK signaling pathway directly or through the induced DNA damage remains unclear. The cisplatin-induced signaling events leading to the activation of ERK seem to include growth factor receptors [30]. However, protein kinase C has also been suggested to be involved in the phosphorylation of ERK by cisplatin [15].

Inhibition of ERK activation results in increased sensitivity to cisplatin-induced cell death in ovarian cancer

cell lines and one melanoma cell line, but in increased resistance against cisplatin in HeLa cells and other melanoma cell lines [5, 15, 19, 23, 25, 30]. None of the described downstream mediators of cisplatin-induced activation of ERK so far explains its divergent cellular effects. It has recently 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 addressed [7, 24]. It has been suggested that ERK may mediate cisplatin-induced accumulation of p53 to trigger apoptosis [21]. However, other forms of DNA damage-induced activation of ERK increase cell death independently of p53 [2, 28].

The p53 tumor suppressor plays a major role in DNA damage-induced growth arrest and apoptosis. Inactivation of the p53 pathway causes resistance to cisplatin [1, 12, 33, 34]. Stabilization of p53 and phosphorylation of serine residues in the N-terminus near the mdm2-binding site following DNA damage regulate the activity of p53. Therefore, the p53 status of tumor cells may determine the diverging cellular consequences of cisplatin-induced activation of ERK.

Here we report that cisplatin-induced activation of ERK is mediated by Ras after short-term treatment. Inhibition of signaling through ERK using either pharmacological inhibitors of MEK1 or tetracycline-regulated expression of a dominant-inhibitory mutant of ERK enhanced cell survival after cisplatin incubation and reduced cisplatin-induced cell death in p53-negative osteosarcoma cells. Induction of p53 using a tetracycline-regulated p53 expression system did not alter the proapoptotic effect of cisplatin-induced activation of ERK.

Materials and methods

Materials

The ERK, JNK and p38 antibodies, anti-phosphospecific ERK and p38 antibodies as well as the anti-phosphospecific p53 antibody were from New England Biolabs (Beverley, Mass.), the anti-phosphospecific JNK antibodies were from Calbiochem (La Jolla, Calif.), the p53 and hemaglutinin (HA) tag antibodies were from Santa Cruz Biotechnology (Santa Cruz, Calif.), UO126 was from Promega (Madison, Wis.) and PD98059 was from Calbiochem. G418 and tetracycline were purchased from Sigma (St. Louis, Mo.). Cisplatin was purchased from ASTA Medica. A stock solution of cisplatin dissolved in 0.9% NaCl was stored at –20°C. The plasmids KRSPA-Ras-N17 and KRSPA were generously provided by Dr. Troppmaier (Wuerzburg, Germany). The plasmid containing dominant-inhibitory HA-ERK1-KR under the control of a tetracycline-responsive element was a gift from Dr. Mivechi (Augusta, Ga.).

Cell lines, culture and transfection

The cell lines Saos-2 (osteosarcoma) and Kelly (neuroblastoma) were obtained from the German Collection of Microorganisms and Cell Cultures (DMSZ). Cells were routinely maintained in RPMI-1640 medium supplemented with 10% (Kelly) or 20% (Saos-2) fetal calf serum (FCS) without antibiotics in a humidified atmosphere containing 5% CO_2 at 37°C. Saos-2 cells containing a reverse tetracycline-controlled transactivator were from BD-Clontech (Heidelberg, Germany) and cultured in RPMI supplemented with 10% FCS and 100 μg/ml G418. They were transfected with the plasmid containing HA-ERK1-KR together with a plasmid transferring puromycin resistance (ratio 1:20) using a lipid-based transfection reagent (lipofectamine plus, Gibco, San Diego, Calif.). After selection with puromycin the resistant Saos-2 cells were tested for the regulated induction of HA-ERK-KR by withdrawal of tetracycline. The cells were called STO-KR and maintained in medium with 100 μg/ml G418, 2 μg/ml puromycin and 2 μg/ml tetracycline. The S-p53-7 cell line is a Saos-2 cell line containing full-length p53 under the control of a tetracycline-regulated promoter [4]. It was routinely maintained in RPMI-1640 supplemented with 10% FCS and 1 μg/ml puromycin, 250 μg/ml G418 and 2 μg/ml tetracycline. KRSPA-Ras-N17 and KRSPA were transiently transfected into Saos-2 cells by calcium phosphate precipitation and treated 48 h later.

Treatment of cells

Cells were grown in 45-mm dishes to 80% confluence for immunoblot studies and seeded at a density of 10^5/ml or 5×10^4/ml in 96-well plates the day prior to therapy for the cytotoxicity or apoptosis assay, respectively. They were treated with the cytotoxic drug for 2 or 24 h. In experiments with PD98059 and UO126, cells were either pretreated for 30 min or treated after cisplatin incubation for a maximum of 24 h as indicated in the Results. The concentration of dimethyl sulfoxide (DMSO) was 0.1%; this concentration neither induced activation of ERK nor changed the cytotoxicity of cisplatin (data not shown). Control cells were treated with DMSO only. After treatment, cells were washed twice with prewarmed phosphate-buffered saline and fresh medium was added. After incubation at 37°C for the indicated times, the appropriate assays were performed. Tetracycline was withdrawn from S-p53-7 cells from 48 h before cisplatin incubation for 72 h to induce the expression of p53 and from STO-KR cells from 24 h before cisplatin incubation for 48 h to induce expression of HA-ERK-KR.

Cytotoxicity assay

The MTT assay, originally developed by Mosmann, was performed with some minor modifications [3, 20]. The relationships between seeding density, incubation volume and incubation period were established for the cell lines after growth studies.

After 4 days of culture, 25 μl MTT (2 mg/ml in 0.9% NaCl) was added to each well and the cells were incubated at 37°C for a further 4 h. The medium was carefully aspirated from the plates. The formazan was solubilized by adding 100 μl 100% DMSO to each well. Immediately after resolubilization, all plates were scanned at 540 nm on a scanning multiwell spectrophotometer. The values from each experiment represent the average of a minimum of four wells.

Apoptosis assay

Apoptosis was assessed by a sandwich ELISA from Roche (Roche Diagnostics, Mannheim, Germany) detecting histone-coupled DNA fragments in the cytoplasm according to the manufacturer's instructions. Briefly, the cytoplasmic fraction of cell lysates from 10^3 cells was incubated for 2 h with biotinylated antibodies directed against histones and peroxidase-coupled anti-DNA antibodies, respectively. After removal of unbound antibodies, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was added as a peroxidase substrate. Absorption was measured at 405 nm (reference 490 nm) with an ELISA reader. The increase in apoptosis relative to untreated cells is shown.

Immunoblotting

After treatment, cells were lysed in sodium dodecyl sulfate (SDS) sample buffer and a fraction from each sample was precipitated

with 10% trichloroacetic acid and the protein concentration was determined by bicinchoninic acid (Pierce, Rockford, Ill.). An equal amount of protein from each sample (30 μg) was resolved by SDS-PAGE using 10% polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat dried milk, washed and incubated with the primary antibodies overnight at 4°C. Following a 1-h incubation with peroxidase-conjugated secondary antibodies proteins were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, N.J.).

Sequence analysis of p53 in Kelly cells

Total RNA was purified using a single-step protocol. The RNA was denatured at 70°C for 5 min and cDNA synthesis was then carried out at 42°C for 60 min in a total volume of 20 μl using random hexamer primers (Roche, Mannheim, Germany). The entire p53 cDNA (accession number K03199) was amplified in two overlapping fragments. For amplification of the first p53 fragment 5′-CTTCCGGGTCACTGCCATGGAG-3′ and 5′-GGGCCTGA CCATCGCTATCTGAG-3′ oligonucleotides were used. The second p53 fragment was amplified with primers 5′-GTGAGGCGCT GCCCCCACCATGAG-3′ and 5′-CTGAGTCAGGCCCTTCT GTCTTG-3′. PCR products were cloned by means of a TA cloning kit (Invitrogen, Groningen, The Netherlands) and four individual clones were sequenced for each fragment.

Results

Cisplatin induces a persistent, Ras-dependent activation of ERK secondary to cisplatin-induced injury

In order to confirm and extend recent data that ERK is activated by cisplatin in tumor cells [5, 15, 21, 23], cultured Saos-2 osteosarcoma cells were exposed to cisplatin for 2 h and activation of ERK was determined by immunoblot analysis using an antibody specific to the phosphorylated form of ERK. ERK was not activated during cisplatin incubation (data not shown). Cisplatin induced a dose-dependent increase in ERK activity starting 3–6 h from the end of cisplatin incubation and declining after 12–24 h (Fig. 1). Cisplatin-induced activation of ERK was also detected during long-term incubation with cisplatin, in a neuroblastoma cell line (Kelly), a Ewing sarcoma cell line (Cado-ES) and K562 cells (data not shown). No activation of SAPK/JNK, but phosphorylation of p38 was detected in Saos-2 cells after short-term cisplatin treatment with concentrations up to 50 μg/ml (data not shown).

We sought to determine whether cisplatin directly activates the ERK signaling cascade or indirectly through induced cellular damage. Indirect evidence from the timing of ERK phosphorylation suggests that the activation may occur after cisplatin-induced damage [23]. To determine whether activation of the ERK signaling cascade is a direct consequence of cisplatin treatment, we inhibited the ERK-activating kinase MEK1 (mitogen-activated protein kinase kinase 1) by PD98059 or UO126 prior to cisplatin incubation [6, 9, 10] and measured ERK phosphorylation by immunoblotting with antiphosphospecific ERK antibodies. Inhibition of MEK1 prior to and during the 2-h cisplatin incubation did not prevent cisplatin-induced ERK activation (Fig. 2A). These results suggest that the ERK signaling cascade may not be activated directly by cisplatin.

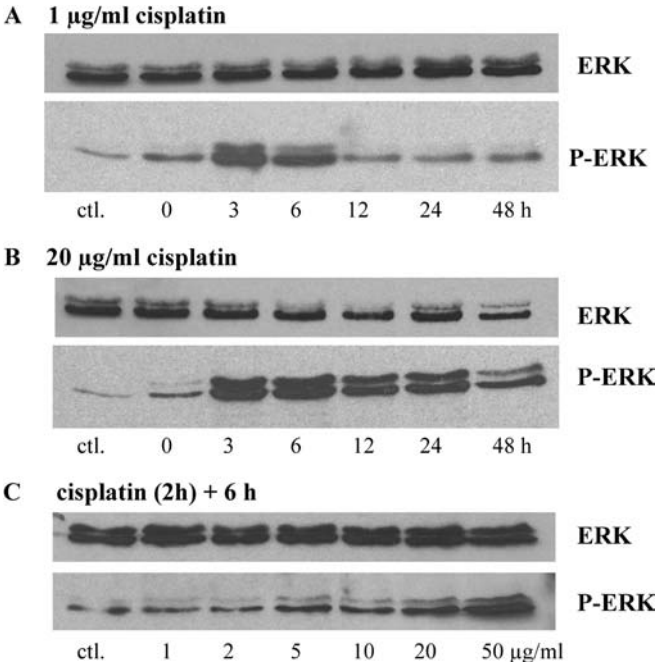

Fig. 1A–C. Cisplatin activates ERK in a time- and dose-dependent manner in Saos-2 cells. **A**, **B** Saos-2 cells were treated with 1 μg/ml (**A**) or 20 μg/ml (**B**) cisplatin for 2 h. At the indicated times thereafter cells were lysed and ERK activation assessed by Western blotting using antibodies to the phosphorylated form of ERK1 and 2. **C** Saos-2 cells were incubated with the indicated concentrations of cisplatin for 2 h followed by 6 h at 37ºC without the drug. ERK activation was again determined by Western blotting

To verify that cisplatin-induced ERK activation occurs after cisplatin-induced cell injury, we inhibited MEK1 at different times after cessation of cisplatin treatment by UO126. ERK activation was not inhibited by incubation with UO126 in the first 2 h after the 2-h cisplatin treatment in Saos-2 cells (Fig. 2A). However, inhibiting MEK1 by UO126 during the third hour after cisplatin treatment completely inhibited ERK activation by cisplatin, as did incubation of the cells with either PD98059 or UO126 during the whole time after cisplatin treatment (Fig. 2B, C). Cells treated with the protein kinase C activator phorbol myristate acetate (PMA) and the MEK1 inhibitors during PMA treatment served as control for the MEK1 inhibitors. These findings suggest that the ERK signaling cascade may be activated by secondary damage induced by cisplatin.

General inhibition of growth factor signaling pathways by suramin as well as antioxidative pretreatment prevents cisplatin-induced ERK activation and apoptosis in HeLa cells [30]. To further define upstream signaling events, we tested the possibility that Ras mediates the activation of ERK by cisplatin. Dominant-negative Ras (Ras-N17) was transiently transfected into Saos-2

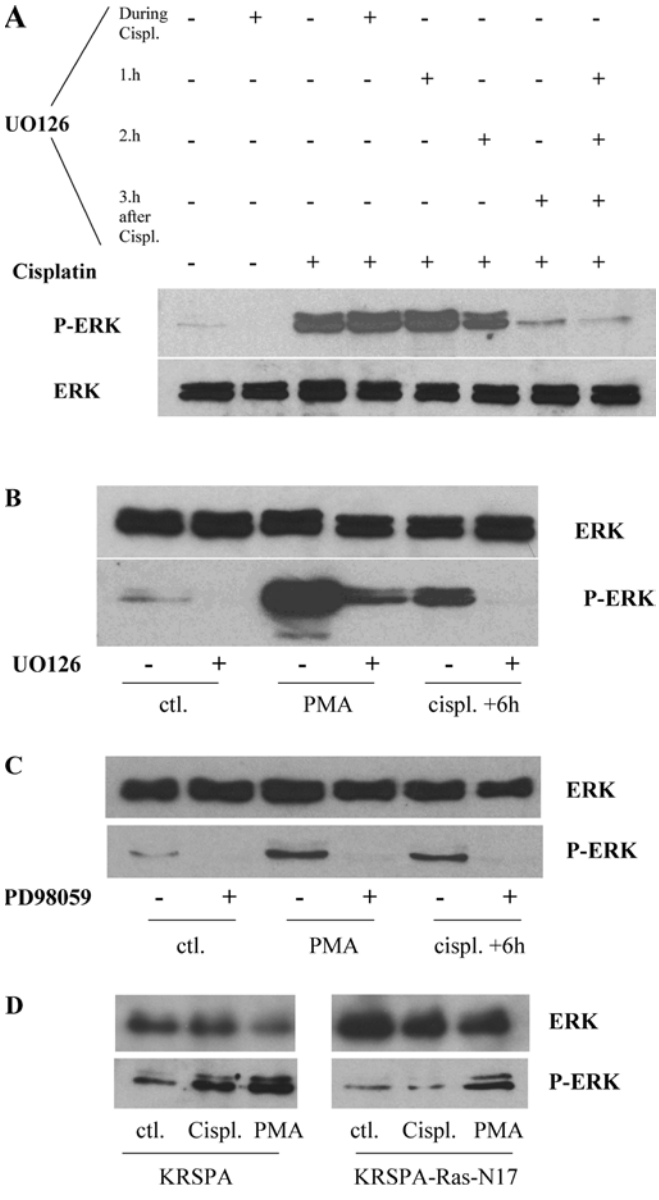

Fig. 2A–D. Activation of ERK occurs Ras-dependently secondary to cisplatin-induced damage. **A–C** Saos-2 cells were treated with 20 μg/ml cisplatin for 2 h. **A** MEK1 was inhibited by 20 μ*M* UO126 during or for 1–3 h after cisplatin incubation. All cells were harvested 3 h after the 2-h cisplatin treatment. **B**, **C** MEK1 was inhibited by 20 μ*M* UO126 for 3 and 6 h after the end of cisplatin incubation. Control cells were incubated with 100 m*M* PMA for 15 min. Activation of ERK was inhibited by 20 μ*M* UO126 (**B**) or 100 μ*M* PD98059 (**C**) after cisplatin treatment or during incubation with PMA. **D** Saos-2 cells were transiently transfected with plasmids KRSPA (control) or KRSPA-Ras-N17 and treated with 20 μg/ml cisplatin for 2 h or 100 n*M* PMA for 15 min 48 h later. ERK activation was determined again by Western blotting 6 h after cisplatin treatment

cells and cisplatin-induced activation of ERK was detected by immunoblotting using antiphosphospecific ERK antibodies. Activation of ERK by cisplatin but not by PMA was inhibited by Ras-N17 (Fig. 2D), but not by the control plasmid KRSPA, suggesting that Ras mediates the phosphorylation of ERK by cisplatin.

Inhibition of cisplatin-induced activation of ERK increases survival and reduces apoptosis of Saos-2 cells

The activation of ERK by different stresses has been suggested to be involved in repair processes helping the cells to survive a potentially lethal treatment [14, 17, 31]. Inhibition of signaling through ERK by inhibition of MEK1 sensitizes some ovarian cell lines and one melanoma tumor cell line to cisplatin-induced cytotoxicity, in accordance with the general view of ERK as a mediator of survival signals [15, 19, 23].

We therefore investigated whether inhibition of ERK activation during or after the 2-h cisplatin exposure sensitized for cisplatin-induced cell death. Inhibition of MEK1 by UO126 for 3 to 24 h after cisplatin treatment did not enhance cellular sensitivity to the drug. In contrast cisplatin-induced cytotoxicity was even reduced (Fig. 3A). This result was confirmed by inhibiting MEK1 with increasing doses of either PD98059 or UO126 for 24 h after cisplatin treatment (Fig. 3B, C) and during long-term (24 h) cisplatin treatment with simultaneous inhibition of MEK1 (Fig. 3D).

Since pharmacological protein kinase inhibitors have a limited specificity we stably transfected a tetracycline-regulated dominant inhibitory mutant of ERK (HA-ERK-KR) into Saos cells (Fig. 3E). Cellular sensitivity against cisplatin was tested with and without genetically inhibiting signaling through ERK using the MTT assay. Overexpression of HA-ERK-KR after withdrawal of tetracycline decreased the cytotoxicity of cisplatin on Saos-2 cells, again confirming that activation of ERK in response to cisplatin leads to an increase in cell death (Fig. 3E).

Recently, it has been shown in HeLa cells that activation of ERK mediates an increase in apoptosis [30]. To determine whether inhibition of MEK1 reduces cisplatin-induced apoptosis, Saos-2 cells were incubated with UO126 or PD98059 for 24 h after the 2-h cisplatin treatment and apoptosis was assessed by an ELISA for the detection of intracytoplasmic DNA fragments. MEK1 inhibition by both UO126 and PD98059 reduced cisplatin-induced apoptosis (Fig. 4).

These results collectively suggest that cisplatin-induced activation of ERK does not enhance resistance to the cytotoxic effects of cisplatin but is related to cisplatin-induced cell death in Saos-2 cells as at is in HeLa cells [30].

p53 accumulation and phosphorylation after cisplatin treatment depends on MEK1 but does not alter the proapoptotic effect of cisplatin-induced activation of ERK

Since ERK has been identified as a p53 kinase even during cisplatin treatment [23, 27] and Saos-2 and HeLa cells do not express p53, we investigated the possibility that the status of p53 determines whether activation of ERK by cisplatin signals to survival or cell death.

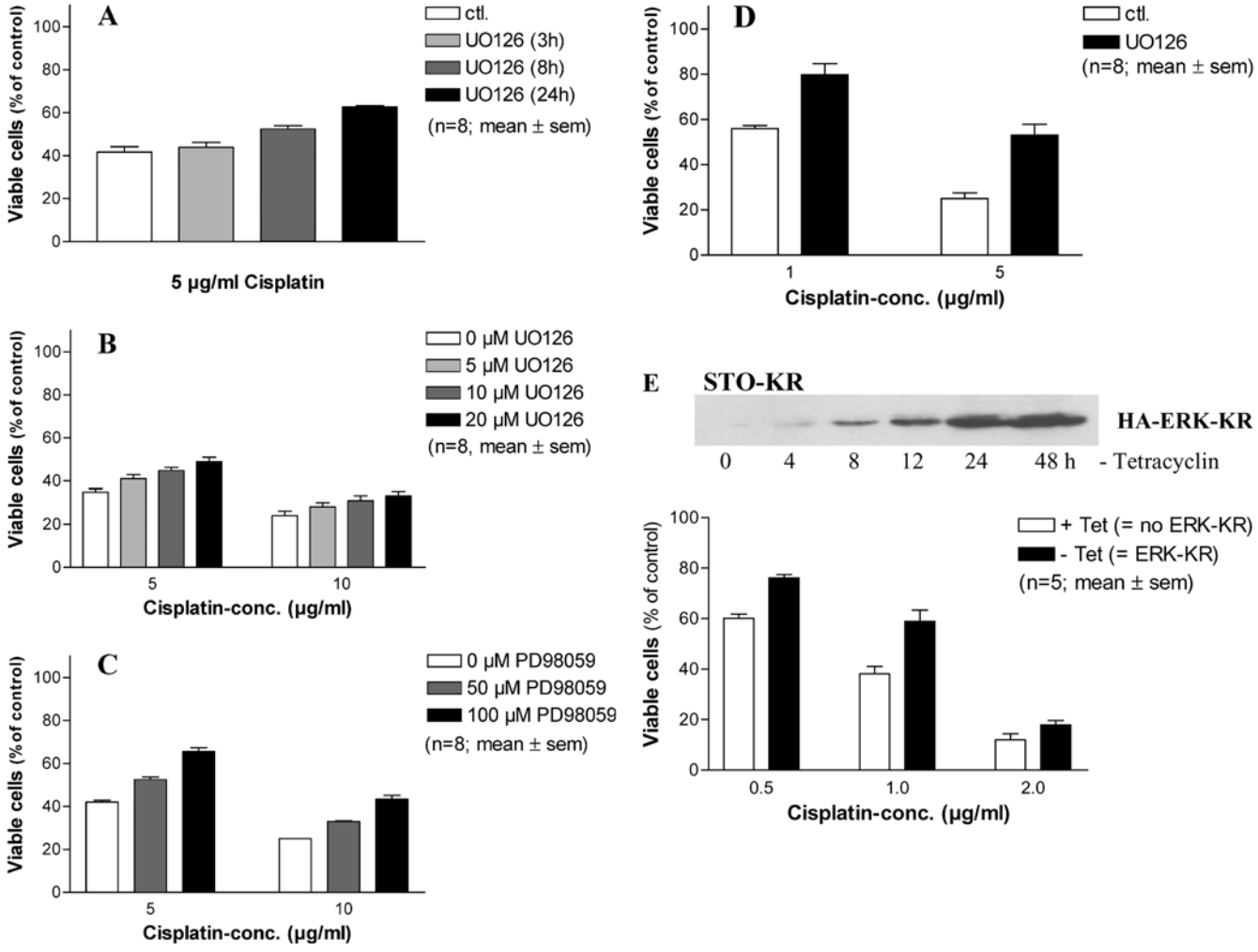

Fig. 3A–E. Inhibition of MEK1 after cisplatin treatment increases survival of Saos-2 cells. **A–C** Saos-2 cells were treated with 5 or 10 µg/ml cisplatin for 2 h followed by treatment with 20 µ*M* UO126 for 3–24 h (**A**) or increasing concentrations of UO126 (**B**) or PD98059 (**C**) for 24 h. Cytotoxicity was determined by the MTT assay. Cell survival is indicated relative to control cells incubated with the MEK1 inhibitor only ($n = 8$, mean ± SEM for each treatment group). **D** Saos-2 cells were treated with 1 or 5 µg/ml cisplatin for 24 h together with 20 µ*M* UO126. Cytotoxicity was assessed by the MTT assay ($n = 8$, means ± SEM). **E** Tetracycline was removed from STO-KR cells for the indicated times and expression of HA-ERK-KR was determined by immunoblotting with an HA antibody (*upper panel*). STO-KR cells were incubated with or without tetracycline from 24 h prior to a 24-h incubation with cisplatin. Cytotoxicity was determined by the MTT assay ($n = 5$, means ± SEM)

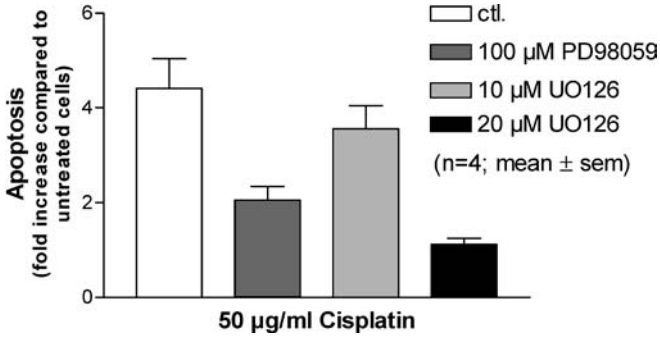

Fig. 4. Inhibition of MEK1 after cisplatin treatment decreases apoptosis of Saos-2 cells. Saos-2 cells were treated with 50 µg/ml cisplatin for 2 h followed by incubation with 100 µ*M* PD98059 or 10 or 20 µ*M* UO126. Apoptosis was assessed by an ELISA for cytoplasmic DNA fragments. Apoptosis is indicated relative to control cells not treated with cisplatin ($n = 4$, means ± SEM)

MEK1-dependent stabilization and phosphorylation of p53 at serine 15 in response to cisplatin was confirmed in the p53-positive neuroblastoma cell line Kelly by immunoblotting with an antiphosphospecific antibody (Fig. 5A). The cytotoxicity of cisplatin in p53-positive Kelly neuroblastoma cells was assessed with and without inhibiting MEK1 by UO126 or PD98059. Inhibition of signaling though ERK increased cell survival in this p53-positive cell line as it did in the p53-negative Saos-2 cells (Fig. 5B). Sequencing revealed no mutation that changed the corresponding amino acid sequence of p53 in Kelly cells. However, the p53-dependent apoptotic pathway might be disrupted in this cell line as well. We therefore analyzed the role of p53 on the proapoptotic effect of cisplatin-induced activation of ERK using a Saos-2 cell line expressing full-length p53 under the

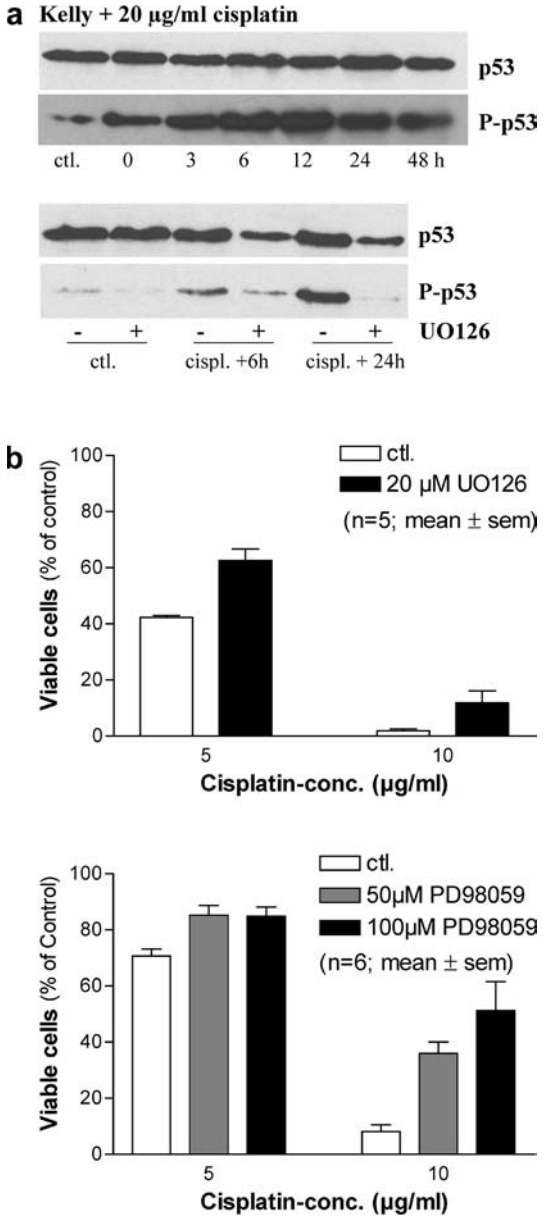

Fig. 5A, B. Inhibition of MEK1 after cisplatin treatment decreases p53 stabilization and phosphorylation and increases survival in p53 positive Kelly cells. **A** p53 is stabilized and phosphorylated in a MEK1-dependent manner at serine 15 after cisplatin incubation. Kelly cells were treated with 20 µg/ml cisplatin for 2 h. At the indicated times thereafter cells were lysed and total p53 was assessed by Western blotting using antibodies specific for p53 or the serine 15 phosphorylated form of p53. MEK1 was inhibited by 20 µ*M* UO126 for 6 and 24 h after the end of the cisplatin incubation and total p53 and phosphorylated p53 were assessed by Western blotting. **B** Kelly cells were treated with 5 or 10 µg/ml cisplatin for 2 h followed by treatment with either UO126 or PD98059 for 24 h. Cytotoxicity was determined by the MTT assay. Cell survival is indicated relative to control cells incubated with the MEK1 inhibitor only ($n = 5$, means ± SEM)

control of a tetracycline-regulated promoter (S-p53-7) [4]. p53 was induced in a time-dependent manner after tetracycline withdrawal in the S-p53-7 cell line (Fig. 6A). Phosphorylation of ERK after cisplatin treatment was induced and was inhibited independently of the p53 status in S-p53-7 cells (Fig. 6B). Inhibition of MEK1 reduced the rate of cisplatin-induced apoptosis independently of the status of p53 in S-p53-7 cells (Fig. 6C). These results suggest that cisplatin-induced accumulation and phosphorylation of p53 is controlled by the ERK signaling pathway. However, expression of p53 does not alter the proapoptotic effect of activated ERK in p53-negative Saos-2 cells compared to Saos-2 cells reconstituted with wild-type p53.

Discussion

Cellular drug resistance against cisplatin can be mediated by altered signal transduction pathways. Activation of the ERK signaling cascade has been implicated in cellular resistance to cisplatin [15, 19, 23, 25] in accordance with the general view of ERK as a mediator of survival signals [14, 17, 31]. Inhibition of signaling through ERK by the MEK1 inhibitors PD98059 or UO126 sensitizes some ovarian cell lines and one melanoma tumor cell line to cisplatin-induced cytotoxicity [15, 19, 23]. In HeLa cells and our osteosarcoma and neuroblastoma cells, however, survival was increased in tumor cells treated with the MEK inhibitors at concentrations and treatment times that inhibit ERK activation [30] (Figs. 3 and 5) and by genetically inhibiting signaling through ERK. These results support recent findings that activation of ERK by means of different cytotoxic agents is involved in the induction of apoptosis and cell cycle arrest [2, 28].

These opposing observations on the biological consequences of activation of the ERK signaling pathway by cisplatin together with data identifying ERK as a p53 kinase even during cisplatin treatment [24, 27] led to the speculation that p53 may determine the cellular fate of cisplatin-induced activation of ERK [21]. Our findings show that the proapoptotic effect of cisplatin-induced activation of ERK is independent of the p53 status of the cells, although the ERK signal transduction pathway takes part in the accumulation and phosphorylation of p53 in response to cisplatin. The resulting model that a p53-independent apoptotic pathway controlled by ERK is activated after genotoxic stress – or a survival signal downregulated by ERK – is supported by recent results on Taxol- and etoposide-induced ERK-dependent apoptosis [2, 28]. This implies that, for the clinical situation, the concept of inhibiting the Ras–ERK signaling pathway [26] together with conventional chemotherapy may not be appropriate for all tumor types. In contrast, tumor survival may even be promoted if Ras–ERK inhibition is applied together with DNA-damaging agents for patients with sarcomas or embryonal tumors independent of the p53 status of the cells.

Our results support the view that cisplatin does not activate the ERK signaling cascade directly, but through the induced damage [23]. Upstream signaling events leading to ERK phosphorylation include Ras-dependent,

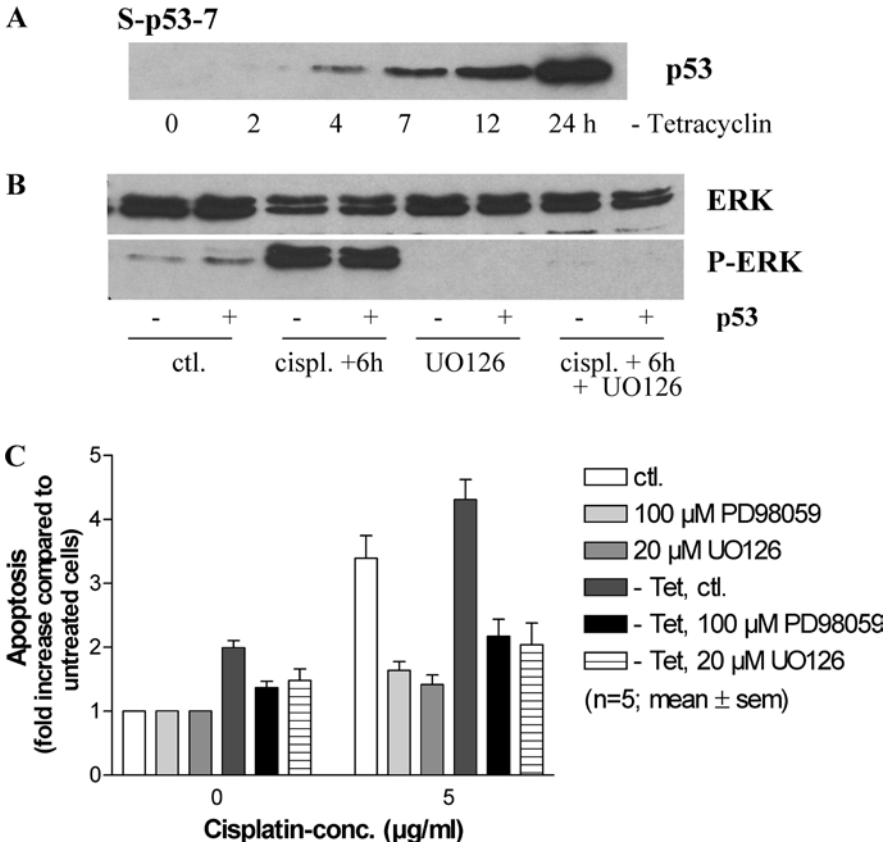

Fig. 6A–C. Expression of p53 does not influence the proapoptotic effect of cisplatin-induced activation of ERK in Saos-2 cells. **A** Tetracycline was removed from S-p53-7 cells for the indicated times and total p53 was determined by immunoblotting. **B** S-p53-7 cells were incubated with or without tetracycline from 2 h prior to a 2-h incubation with cisplatin. MEK1 was inhibited by 20 μM UO126 after cisplatin treatment as indicated. Cells were lysed 6 h after the incubation with cisplatin and ERK phosphorylation was determined by immunoblotting. **C** S-p53-7 cells were incubated with or without tetracycline from 72 h before a 24-h cisplatin incubation. MEK1 was inhibited by 100 μM PD98059 or 20 μM UO126 for 24 h together with the cisplatin treatment, and apoptosis was assessed by the ELISA for cytoplasmic DNA fragments ($n = 5$, means ± SEM)

PKC-dependent or sphingosine-induced phosphorylation of Raf1 and MEK1. General inhibition of growth factor signaling pathways by suramin as well as antioxidative pretreatment prevent cisplatin-induced ERK activation and apoptosis in HeLa cells [30]. Together with earlier observations that the cytotoxicity of cisplatin is associated with generation of reactive oxygen species, these findings form the basis for a model presented by Wang et al. that oxidants generated in response to cisplatin activate growth factor receptors followed by Ras-mediated activation of Raf-MEK1 and ERK [30]. Our finding that dominant-negative Ras inhibits ERK phosphorylation induced by cisplatin provides further evidence for the involvement of a Ras-dependent pathway in cisplatin-induced activation of ERK.

The immediate downstream signaling events of cisplatin-induced activation of ERK leading to the induction of p53 independent cell death are unknown. Prolonged or constitutive activation of the ERK signaling pathway triggers in some cell systems p53-independent expression of $p21^{WAF1}$ and GADD45 also by DNA damage which in turn could be connected to cell cycle arrest and apoptosis [11, 28, 29, 32]. Whether $p21^{WAF1}$ constitutes a component of the cisplatin-induced apoptotic response mediated by the ERK signaling cascade is the subject of further studies.

To summarize, ERK activation is Ras-dependent after cisplatin-induced cellular damage. ERK activation increased cisplatin-induced cell death independently of p53 in a neuroblastoma cell line and an osteosarcoma cell line. Further work is needed to characterize the molecular background of the cell type specificity of this proapoptotic effect as well as the downstream signaling events connecting the activation of ERK in response to cisplatin to the initiation of apoptosis.

Acknowledgements We are indebted to Mrs. Simone Schwalm for excellent technical assistance. We thank Dr. Troppmaier (Wuerzburg, Germany) for providing the plasmid containing Ras-N17 and Dr. Mivechi (Augusta, Ga.) for providing the plasmid HA-ERK-KR. This work was supported by the "Novartis-Stiftung für therapeutische Forschung" (Nuernberg, Germany).

References

1. Anthoney DA, McIlwrath AJ, Gallagher WM, Edlin AR, Brown R (1996) Microsatellite instability, apoptosis, and loss of p53 function in drug- resistant tumor cells. Cancer Res 56:1374
2. Bacus SS, Gudkov AV, Lowe M, Lyass L, Yung Y, Komarov AP, 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
3. Carmichael J, DeGraff WG, Gazdar AF, Minna JD, Mitchell JB (1987) Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. Cancer Res 47:936
4. Chen X, Ko LJ, Jayaraman L, Prives C (1996) p53 levels, functional domains, and DNA damage determine the extent of the apoptotic response of tumor cells. Genes Dev 10:2438

5. Cui W, Yazlovitskaya EM, Mayo MS, Pelling JC, Persons DL (2000) Cisplatin-induced response of c-jun N-terminal kinase 1 and extracellular signal-regulated protein kinases 1 and 2 in a series of cisplatin-resistant ovarian carcinoma cell lines. Mol Carcinog 29:219
6. Davies SP, Reddy H, Caivano M, Cohen P (2000) Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem J 351:95
7. DeHaan RD, Yazlovitskaya EM, Persons DL (2001) Regulation of p53 target gene expression by cisplatin-induced extracellular signal-regulated kinase. Cancer Chemother Pharmacol 48:383
8. Dempke W, Voigt W, Grothey A, Hill BT, Schmoll HJ (2000) Cisplatin resistance and oncogenes – a review. Anticancer Drugs 11:225
9. Dudley DT, Pang L, Decker SJ, Bridges AJ, Saltiel AR (1995) A synthetic inhibitor of the mitogen-activated protein kinase cascade. Proc Natl Acad Sci U S A 92:7686
10. Duncia JV, Santella JB, Higley CA, Pitts WJ, Wityak J, Frietze WE, Rankin FW, Sun JH, Earl RA, Tabaka AC, Teleha CA, Blom KF, Favata MF, Manos EJ, Daulerio AJ, Stradley DA, Horiuchi K, Copeland RA, Scherle PA, Trzaskos JM, Magolda RL, Trainor GL, Wexler RR, Hobbs FW, Olson RE (1998) MEK inhibitors: the chemistry and biological activity of U0126, its analogs, and cyclization products. Bioorg Med Chem Lett 8:2839
11. Fanton CP, McMahon M, Pieper RO (2001) Dual growth arrest pathways in astrocytes and astrocytic tumors in response to Raf-1 activation. J Biol Chem 15:15
12. Gallagher WM, Cairney M, Schott B, Roninson IB, Brown R (1997) Identification of p53 genetic suppressor elements which confer resistance to cisplatin. Oncogene 14:185
13. Giaccone G (2000) Clinical perspectives on platinum resistance. Drugs 59:9
14. Guyton KZ, Liu Y, Gorospe M, Xu Q, Holbrook NJ (1996) Activation of mitogen-activated protein kinase by H_2O_2. Role in cell survival following oxidant injury. J Biol Chem 271:4138
15. 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
16. Jordan P, Carmo-Fonseca M (2000) Molecular mechanisms involved in cisplatin cytotoxicity. Cell Mol Life Sci 57:1229
17. Kasid U, Pfeifer A, Brennan T, Beckett M, Weichselbaum RR, Dritschilo A, Mark GE (1989) Effect of antisense c-raf-1 on tumorigenicity and radiation sensitivity of a human squamous carcinoma. Science 243:1354
18. Kelland LR (2000) Preclinical perspectives on platinum resistance. Drugs 59:1
19. Mandic A, Viktorsson K, Heiden T, Hansson J, Shoshan MC (2001) The MEK1 inhibitor PD98059 sensitizes C8161 melanoma cells to cisplatin-induced apoptosis. Melanoma Res 11:11
20. Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65:55
21. Park SA, Park HJ, Lee BI, Ahn YH, Kim SU, Choi KS (2001) Bcl-2 blocks cisplatin-induced apoptosis by suppression of ERK-mediated p53 accumulation in B104 cells. Brain Res Mol Brain Res 93:18
22. Perez RP (1998) Cellular and molecular determinants of cisplatin resistance. Eur J Cancer 34:1535
23. Persons DL, Yazlovitskaya EM, Cui W, Pelling JC (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
24. Persons DL, Yazlovitskaya EM, Pelling JC (2000) Effect of extracellular signal-regulated kinase on p53 accumulation in response to cisplatin. J Biol Chem 275:35778
25. Sanchez-Perez I, Murguia JR, Perona R (1998) Cisplatin induces a persistent activation of JNK that is related to cell death. Oncogene 16:533
26. Sebolt-Leopold JS, Dudley DT, Herrera R, Van Becelaere K, Wiland A, Gowan RC, Tecle H, Barrett SD, Bridges A, Przybranowski S, Leopold WR, Saltiel AR (1999) Blockade of the MAP kinase pathway suppresses growth of colon tumors in vivo. Nat Med 5:810
27. Shih A, Lin HY, Davis FB, Davis PJ (2001) Thyroid hormone promotes serine phosphorylation of p53 by mitogen-activated protein kinase. Biochemistry 40:2870
28. Tang D, Wu D, Hirao A, Lahti JM, Liu L, Mazza B, Kidd VJ, Mak TW, Ingram AJ (2002) ERK activation mediates cell cycle arrest and apoptosis after DNA damage independently of p53. J Biol Chem 30:30
29. Tong T, Fan W, Zhao H, Jin S, Fan F, Blanck P, Alomo I, Rajasekaran B, Liu Y, Holbrook NJ, Zhan Q (2001) Involvement of the MAP kinase pathways in induction of GADD45 following UV radiation. Exp Cell Res 269:64
30. Wang X, Martindale JL, Holbrook NJ (2000) Requirement for ERK activation in cisplatin-induced apoptosis. J Biol Chem 275:39435
31. Woessmann W, Meng YH, Mivechi NF (1999) An essential role for mitogen-activated protein kinases, ERKs, in preventing heat-induced cell death. J Cell Biochem 74:648
32. Woods D, Parry D, Cherwinski H, Bosch E, Lees E, McMahon M (1997) Raf-induced proliferation or cell cycle arrest is determined by the level of Raf activity with arrest mediated by p21Cip1. Mol Cell Biol 17:5598
33. Yazlovitskaya EM, DeHaan RD, Persons DL (2001) Prolonged wild-type p53 protein accumulation and cisplatin resistance. Biochem Biophys Res Commun 283:732
34. Zamble DB, Jacks T, Lippard SJ (1998) p53-dependent and -independent responses to cisplatin in mouse testicular teratocarcinoma cells. Proc Natl Acad Sci U S A 95:6163