

High Level of Mitogen-Activated Protein Kinase Phosphatase-1 Expression Is Associated With Cisplatin Resistance in Osteosarcoma

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Background. Cisplatin is one of the most effective chemotherapeutic agents in the treatment of several solid tumors including osteosarcoma (OS). Despite aggressive treatment, 25% of patients with OS continue to die from their disease. Since cisplatin based regimens have been uniformly used in OS therapy, treatment failure is likely due, at least in part, to cisplatin resistance. **Procedure.** The objective of this study was to determine the relationship between MKP-1 expression and cisplatin sensitivity of OS cell lines and to explore the mechanism underlying this relationship. Three OS cell lines were examined for their MKP-1 expression and cisplatin sensitivity. JNK phosphorylation and apoptosis induction was also measured. Western and Northern blot, flow cytometry, siRNA, and MTT assays were used. **Results.** U2OS cells, which express high

level of MKP-1, are less sensitive to cisplatin-induced cell death. Inhibition of MKP-1 by siRNA silencing sensitizes U2OS cells to cisplatin-induced cell death. Furthermore, delayed apoptosis induction following cisplatin treatment was observed in U2OS, in parallel to decreased JNK activation, increased MKP-1 expression and relatively increased cisplatin resistance. Interestingly, triptolide, an MKP-1 inhibitor, blocks MKP-1 expression and enhances cisplatin-induced cell death. **Conclusion.** High MKP-1 expression is associated with decreased sensitivity or increased resistance to cisplatin-induced cell death in OS cell lines, and MKP-1 could potentially be used as a marker of cisplatin resistance and a therapeutic target for molecular therapies. *Pediatr Blood Cancer* 2008;51:754–759. © 2008 Wiley-Liss, Inc.

Key words: chemotherapy; cisplatin; drug resistance; MKP-1; osteosarcoma

INTRODUCTION

Cisplatin is one of the most effective chemotherapeutic agents and is widely used in the treatment of a variety of pediatric and adult solid tumors including osteosarcoma (OS), medulloblastoma, and neuroblastoma. Cisplatin is a DNA-damaging agent that forms cisplatin-DNA adducts and kills cells via several mechanisms, including induction of apoptosis [1]. Resistance to cisplatin is multifactorial and leads to poor response to chemotherapy and treatment failure. Failure to induce apoptosis is believed to be one of the major mechanisms underlying cisplatin resistance [2,3].

OS is the most common malignant bone tumor in children. The introduction of chemotherapy in 1970s significantly increased the long-term survival rate of OS from 15% to 70% [4]. However, 30–40% of patients with OS relapse with current therapies. After relapse, only approximately one-quarter of patients survive beyond 5 years [5]. In the past several years, only minor improvements have been achieved by intensifying chemotherapy, frequently offset by significantly increasing toxicity. Efforts at identifying new risk factors for the stratification of OS treatment are ongoing [5–7]. Histological response after neoadjuvant chemotherapy assessed at the time of definitive surgery is the only reliable marker that can predict outcome [2,3,8]. Since cisplatin is one of the most effective chemotherapeutic agents that has been uniformly used in front line chemotherapy of OS, the poorer response to OS chemotherapy is likely due, at least in part, to cisplatin resistance.

It has been shown that cisplatin can activate the mitogen-activated protein kinase (MAPK) pathways, leading to apoptotic cell death [9]. There are three major pathways of MAPKs: stress-activated protein kinase (SPK)/c-Jun NH2-terminal kinase (JNK), p38 MAPK, and extra-cellular signal-regulated kinase (ERK) [10]. The importance of MAPK activation in cellular response to cisplatin has been gradually appreciated in recent years [11,12]. MAPKs control cell proliferation, differentiation, and cell death. Their role in response to cisplatin is complex and the response varies with the type of cells as well as their proliferation and differentiation status [11–14]. It is believed that MAPK activities are regulated through

reversible phosphorylation of both threonine and tyrosine residues by upstream kinases, as well as by the members of MAPK phosphatase (MKP) family, which can dephosphorylate both phospho-threonine and phospho-tyrosine residues and inactivate MAPKs [2,15]. Recent studies suggest that MKP-1, the founding member of MKP family, plays an important role in the response of cancer cells to chemotherapy, especially to cisplatin [11,14,16]. Therefore, further investigation is warranted to study the role of MKP-1 in pediatric cancers including OS.

MATERIALS AND METHODS

Reagents

Cisplatin was the product of Sicor Pharmaceuticals (Irvine, CA). Triptolide was purchased from Calbiochem (Darmstadt, Germany). MKP-1 antibody was the product of Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against total and phosphorylated JNK (p-JNK) and PARP, were the products of Cell Signaling Technology (Beverly, MA). Anti-actin antibody (AC-74) was purchased from Sigma.

Cell Lines and Cell Cultures

The human OS cell line U2OS, M189 and P16T were as previously described [17,18]. These cells were maintained in RPMI

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1640 medium containing 10% of fetal bovine serum (FBS) (Hyclone, Logan, UT), and 1% penicillin–streptomycin (Invitrogen Life Technologies, Carlsbad, CA) in a humidified atmosphere of 5% CO₂ at 37°C.

Polyacrylamide Gel Electrophoresis (PAGE) and Western Blotting

Whole-cell lysates were prepared as described [14]. Cell lysates (30–100 µg) were electrophoresed through 10–15% SDS–PAGE. Following electrophoresis, separated proteins on gels were electrotransferred to PVD membranes (Schleicher & Schuell, Inc., Keene, NH). Non-specific binding was blocked with blocking buffer containing 10% fat-free milk powder in Tris buffer saline (TBS) for 1 hr at room temperature, followed by incubation with primary antibodies in blocking buffer with 5% of fat-free milk powder overnight at 4°C and room temperature for 1 hr. The membrane was then washed three times with TBS, and incubated with secondary antibody for 1 hr at room temperature and washed three times. The bound antibody was detected using Chemiluminescence reagent (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's protocol.

Isolation of RNA and Northern Blot Analysis

Total cellular RNA was purified using the Trizol reagent (Invitrogen Life Technologies) according to the manufacturer's instructions. Total RNA was separated in a 1.5% formaldehyde agarose gel and blotted to Hybond-N+ membrane (Amersham Pharmacia Biotech). The blots were hybridized with ³²P labeled human MKP-1 cDNA as described previously [14]. Radioactive signals were analyzed by autoradiography.

siRNA Assay

MKP-1 siRNA pooled oligonucleotides were purchased from Dharmacon Research (Lafayette, CO). Briefly, U2OS cells were plated at 10⁵ cells per well in 6-well plates and transfected with MKP-1 siRNA using Oligofectamine reagent (Invitrogen Life Technologies) according to the manufacturer's instruction and as described previously [14]. siGlow, which is Lamin A siRNA, was used as a non-target fluorescent control. The cells were harvested 48–72 hr after transfection for examination of MKP-1 expression by Western blot and determination of cisplatin sensitivity by MTT assays.

MTT Assays

MTT assays were performed as described previously [14]. Briefly, cells were treated with cisplatin at different concentrations for various times. In the experiments involving the triptolide, cells were pretreated with triptolide for 2 hr before the cisplatin treatment. After incubation with MTT solution, acid isopropanol was added to dissolve formazan crystals. Absorbance was measured using a Vmax microplate reader (Molecular Devices, Sunnyvale, CA) at 590 nm, and percentage of survival was calculated.

In Vitro Cytotoxicity Assay Using Annexin V/PI and Flow-Count Fluorospheres

P16T and U2OS cells were incubated in the presence of 5 µg/ml of cisplatin for 4, 16, 20, and 24 hr in 35 mm Petri dishes. The

experiment was designed to add the cisplatin at the different time points so that the cells could be harvested and assayed at the same time to minimize variation. At the end of treatment, the cisplatin treated and control cells (with no drug added), which served as a baseline control for spontaneous apoptosis, were trypsinized and resuspended in medium. Apoptosis detection was performed using the Apoptosis Annexin-V FITC Kit (Beckman Coulter, Brea, CA) per manufacturer's instruction and as previously described [19,20]. Briefly, 50 µl of resuspended cells were transferred to clean 12 × 75 culture tubes containing 50 µl of annexin V FITC/PI reagent and incubated in the dark for 15 min. At the end of incubation, an additional 0.4 ml 1X Binding Buffer containing 2.5–5% by volume Flow-Count Fluorospheres (Beckman Coulter) was added to each tube, vortexed and then analyzed using a Coulter XL Flow Cytometer (Coulter, Miami, FL). Early apoptotic events were based on the annexin V positive, PI negative population.

Statistical Analysis

All data are expressed as the mean ± SEM. Statistical analyses were done using Student's *t*-test. The data are presented as the mean ± SD, and *P* ≤ 0.05 was considered significant.

RESULTS

High MKP-1 Expressing OS Cells Are Relatively More Resistant to Cisplatin

Three OS cell lines, P16T, M189 and U2OS, were analyzed for their level of MKP-1 expression and cisplatin sensitivity (Fig. 1A–D). U2OS cells, which expressed highest level of MKP-1, were most resistant to cisplatin-induced cell death. On the other hand, only minimal MKP-1 expression was detected in P16T cells which were most sensitive to cisplatin-induced cell death (Fig. 1A–D). The above association of high MKP-1 expression and cisplatin resistance implies that MKP-1 could be an important determinant of cisplatin resistance. Of note, the concentrations of cisplatin used in Figure 1C were across the *in vivo* concentration spectrum when 100 mg/m² of cisplatin was administered to patients as a 24-hr intravenous infusion [21,22]. Because higher concentrations of cisplatin are required for the siRNA assay and for induction of JNK activation as previously reported [14], we also used cisplatin at higher than clinically applicable concentrations for 24-hr MTT assays. Again, U2OS cells were more resistant to cisplatin than P16T cells (Fig. 1D).

siRNA Silencing of MKP-1 Increases Cisplatin Sensitivity in OS

Since high MKP-1 expressing OS cells are relatively more resistant to cisplatin, we hypothesize that decreasing MKP-1 expression will render the cells less resistant. To test this hypothesis, the effect of MKP-1 knock down by siRNA on cisplatin sensitivity was examined. U2OS cells, which are relatively more resistant to cisplatin and express higher levels of MKP-1, were transfected with MKP-1 siRNA (Fig. 2). The results showed that transfection with MKP-1 siRNA significantly inhibited MKP-1 expression, indicating the silencing effect of MKP-1 siRNA (Fig. 2A). As hypothesized, MKP-1 knock down significantly increased the cisplatin-induced cytotoxicity (Fig. 2B). As knock down of MKP-1 renders OS cells less resistant to cisplatin, it is likely that

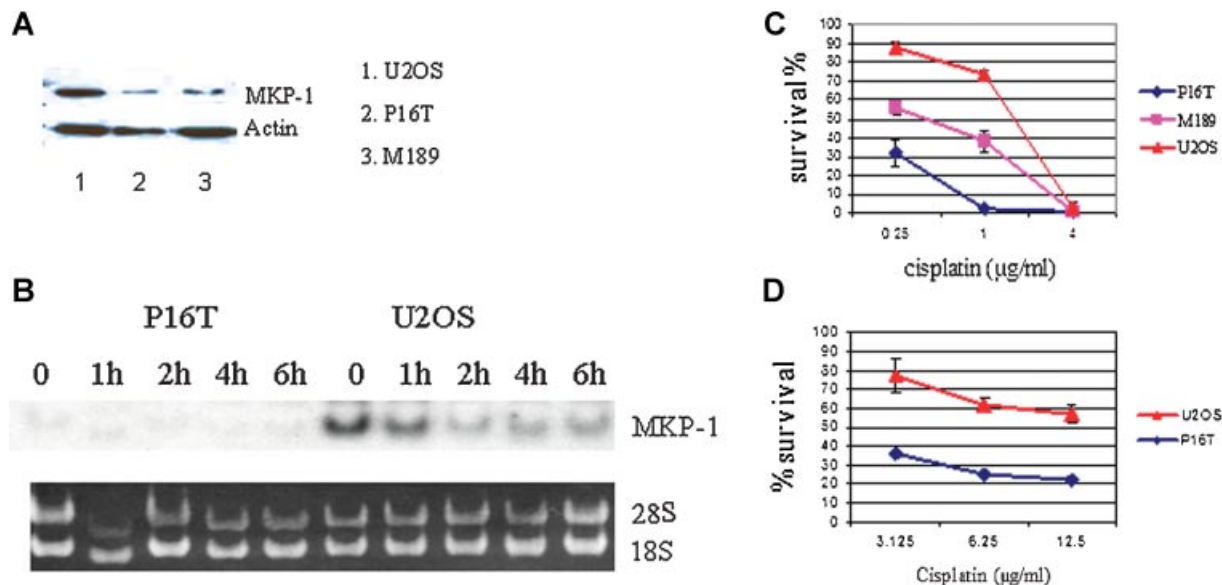


Fig. 1. MKP-1 expression and cisplatin sensitivity in OS cell lines. **A:** MKP-1 expression in three OS cell lines as determined by Western blot. The high level of MKP-1 expression was evident in U2OS cells, and only minimal MKP-1 expression was detected in P16T cells. Actin was a loading control. **B:** MKP-1 expression determined by Northern blot. U2OS and P16T cells were treated with 50 µg/ml of cisplatin for 1, 2, 4, and 6 hr. MKP-1 expression was detected in U2OS, but not in P16T cells prior to and following cisplatin treatment. 18S and 28S were loading controls. **C,D:** Cisplatin-induced cell death in OS cell lines. The OS cells were incubated in the presence of cisplatin for 72 hr (C) at indicated concentrations across the pharmacologically achievable in vivo concentration spectrum. The cell survivals were determined by MTT assay. When OS cells were treated with 1 µg/ml of cisplatin, almost all P16T cells were killed after 72 hr, but 74% of U2OS cells survived the treatment (C). U2OS cells were also more resistant than P16T cells when the cells were incubated in the higher concentrations of cisplatin for 24 hr (D). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

MKP-1 plays an important role in cisplatin resistance. Of note, to catch the peak effect of transient MKP-1 knock down by siRNA, the 24-hr MTT assay with cisplatin at higher than clinically applicable concentrations was used in this experiment to demonstrate a significant difference between the control and siRNA transfected cells.

Cisplatin-Induced Cell Death Is Associated With Apoptosis

To further delineate the mechanisms of cisplatin-induced cytotoxicity and cisplatin resistance in OS, we examined PARP cleavage by Western blot and annexin V staining by flow

cytometry as previously described [19,20]. As shown in Figure 3A and B, the increased percentage of annexin V positive/PI negative cell population and increased PARP cleavage were observed following cisplatin treatment. In addition, both PARP cleavage and percentage of annexin V positive/PI negative cell population were increased more robustly and earlier in the time course in P16T cells compared to U2OS cells. These results suggest that cisplatin-induced cell death is associated with apoptosis induction, and the delay of apoptosis induction in U2OS cells is associated with relative cisplatin resistance. Furthermore, increased apoptosis in P16T cells is associated with increased cisplatin sensitivity.

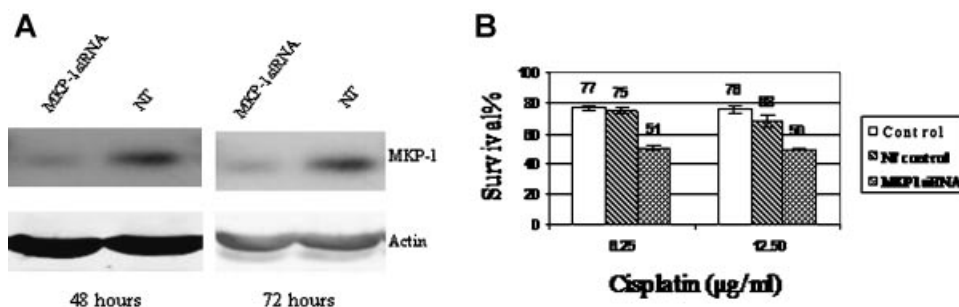


Fig. 2. The effect of MKP-1 knock down on cisplatin sensitivity in U2OS cells. U2OS cells were transfected with MKP-1 siRNA. Forty-eight and 72 hr after transfection, MKP-1 expression was determined by Western blot and cisplatin cytotoxicity measured by MTT assay. MKP-1 siRNA and NT represent MKP-1 siRNA and non-target transfected cells respectively; Control represents U2OS cells without transfection. **A:** Significantly decreased MKP-1 expression was observed at both 48 and 72 hr after transfection of MKP-1 siRNA. Actin was a loading control. **B:** Significantly increased cisplatin induced cell death was observed in MKP-1 siRNA transfected cells. At 48 hr after siRNA transfection, the cells were treated with 6.25 and 12.5 µg/ml of cisplatin for 24 hr.

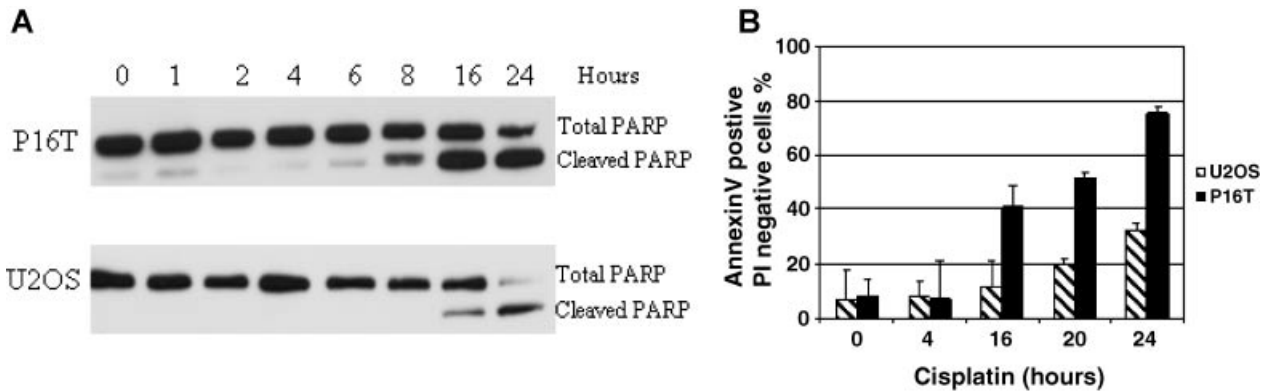


Fig. 3. Cisplatin-induced apoptosis in OS cells: P16T and U2OS cells were treated with 5 $\mu\text{g/ml}$ of cisplatin for the indicated time, and the cells with no drug added (0 hr) served as controls. The total and cleaved PARP was determined by Western blot analysis, annexin V/PI staining was assayed by flow cytometry. **A:** Increased cleavage of PARP is evident 8 hr following cisplatin treatment in P16T cells but not until 16 hr in U2OS cells. **B:** Significantly increased percentage of annexin V positive/PI negative cell population was obvious 16 hr following cisplatin treatment in P16T cells, but not until 20 hr in U2OS cells. In addition, the more robust increase in annexin V positive/PI negative cell population was observed in P16T compared to U2OS cells.

Cisplatin Induces Apoptosis by Activating JNK Pathways and Expression of MKP-1 Is Associated With Delay of Both JNK Phosphorylation and Apoptosis Induction

To determine the relationship between cisplatin-induced JNK activation and cell death in OS, we evaluated the phosphorylation status of JNK and cleavage of PARP in response to cisplatin treatment in U2OS and P16T cells. Delay of JNK phosphorylation as well as PARP cleavage was observed in U2OS cells when compared to P16T cells (Fig. 4). This suggests that delayed JNK activation is associated with delayed apoptosis induction and relative resistance to cisplatin in U2OS cells.

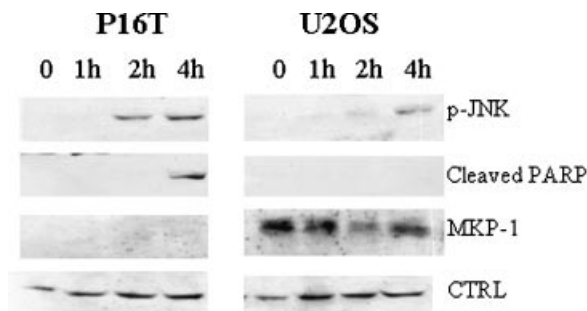


Fig. 4. JNK activation and PARP cleavage following cisplatin treatment. P16T and U2OS cells were treated with cisplatin at 50 $\mu\text{g/ml}$ for 1, 2, and 4 hr. JNK phosphorylation (p-JNK) was undetectable in both P16T and U2OS cells prior to cisplatin treatment, but became evident at 2 hr after cisplatin treatment in P16T cells followed by PARP cleavage at 4 hr. In U2OS cells, p-JNK was not observed until 4 hr after cisplatin treatment and no PARP cleavage detected. MKP-1 expression was detected throughout the course in U2OS but not in P16T cells. p-JNK, cleaved PARP and MKP-1 were determined by Western blot. CTRL was a loading control.

Inhibition of MKP-1 Expression by Triptolide Enhances Cisplatin-Induced Cell Death in OS Cells

Triptolide has been reported to inhibit MKP-1 expression in immortalized murine alveolar macrophages and macrophage cell line RAW 264.7 [23,24]. However, its effect on the MAPK pathways in tumor cells has not been previously studied. We have demonstrated that MKP-1 silencing by siRNA enhances cisplatin-induced cell death and renders U2OS cells more sensitive to cisplatin (Fig. 2). We expect that triptolide, by inhibiting MKP-1 expression, may enhance cisplatin-induced cell death in high MKP-1-expressing OS cells. To this end, MKP-1 expression, prior to and following triptolide treatment, in U2OS cells was examined. While MKP-1 expression was evident before triptolide treatment in U2OS cells, it became undetectable after 4 hr of treatment (Fig. 5A). This indicates that triptolide is a potent MKP-1 inhibitor in OS cells. We then compared cisplatin-induced cell death in the presence and absence of triptolide. Indeed, significantly increased cytotoxicity was observed with the combination of cisplatin and triptolide compared to cisplatin alone (Fig. 5B). These findings again suggest that MKP-1 inhibition may enhance cisplatin-induced cell death in OS.

DISCUSSION

Cisplatin is effective against a variety of solid tumors including OS, neuroblastoma, and medulloblastoma. One of the drawbacks of cisplatin treatment is development of resistance. The mechanism underlying this resistance is incompletely understood. In the current study, we have chosen OS as a model to study cisplatin resistance because of the important role cisplatin plays in the treatment of OS. We showed that MKP-1 high-expressing U2OS cells are relatively more resistant to cisplatin-induced cell death compared to MKP-1 low-expressing P16T cells. The association of high MKP-1 expression and increased cisplatin resistance implies that MKP-1 could be an important determinant of cisplatin resistance. However, since each cell line may have different genetic alterations that could

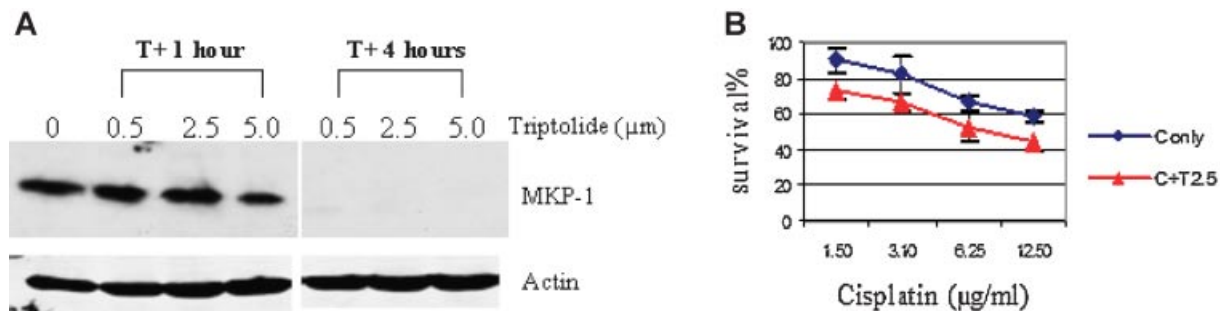


Fig. 5. MKP-1 expression and cisplatin cytotoxicity following triptolide treatment. **A:** MKP-1 expression following triptolide treatment. U2OS cells were incubated in the presence of triptolide at 0.5, 2.5, and 5.0 μM for 1 and 4 hr. While MKP-1 expression was essentially unchanged after 1 hr of triptolide treatment, it became undetectable after 4 hr of treatment. Decreased MKP-1 level was also observed after 2 hr of treatment with 2.5 μM of triptolide (data not shown). Actin was a loading control. **B:** Cisplatin cytotoxicity following triptolide treatment. U2OS cells were first incubated in the presence of 2.5 μM of triptolide or medium. Two hours later, the triptolide-containing medium was replaced with fresh medium and cisplatin was added at indicated concentrations for a 24-hr MTT assay. Increased cisplatin cytotoxicity was observed when U2OS cells were exposed to the combination of triptolide and cisplatin compared to cisplatin alone. C only: Cisplatin alone, C + T 2.5: cisplatin and 2.5 μM of triptolide. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

also affect cisplatin sensitivity, the relationship of MKP-1 expression and cisplatin sensitivity in different cell lines may not fully reflect the role of MKP-1 expression in cisplatin resistance. To this end, MKP-1 siRNA silencing in U2OS cells was performed to minimize the effect of other genetic variations on cisplatin sensitivity. The results showed that MKP-1 knockdown sensitizes OS cells to cisplatin-induced cell death. Furthermore, decreased MKP-1 expression and increased cisplatin-induced cell death was observed when the OS cells were pre-treated with triptolide, a recently identified MKP-1 inhibitor. These data suggest that high level of MKP-1 expression contributes to cisplatin resistance of OS.

MKP-1 dephosphorylates JNK and negatively regulates its activity [11,25]. Since JNK has been shown to be an important mediator of cisplatin-mediated killing, it has been postulated that inhibition of JNK activation is one of the mechanisms underlying the MKP-1 mediated cisplatin resistance [25]. The results of current study are in support of that hypothesis, as high MKP-1 expression in U2OS cells is associated with delayed JNK activation and apoptosis induction alone with decreased cytotoxicity induced by cisplatin.

We have used clinically applicable concentrations of cisplatin in the current study to measure cisplatin-induced cell death and apoptosis induction in order to mimic *in vivo* condition as much as possible. However, no persistent JNK activation was observed when the cells were treated with 5 $\mu\text{g/ml}$ of cisplatin, therefore, a higher concentration was used for these experiments as previously described in the literature [14]. In order not to miss the peak effect of transient knock down of MKP-1 expression in the siRNA assay, higher concentrations of cisplatin were also used to demonstrate difference in cisplatin-induced killing between the control and transfected cells in a 24-hr MTT assay.

In summary, we have shown that high MKP-1 expressing OS cells are more resistant to cisplatin and MKP-1 inhibition enhances cisplatin-induced cell death. Therefore, MKP-1 could potentially be used as a marker of cisplatin resistance, and the combination of MKP-1 inhibitors and cisplatin could be a novel strategy to overcome cisplatin resistance. The current study is focused on OS; however, the result may have implication beyond OS based on the broad application of cisplatin in the treatment of other pediatric and adult solid tumors, such as neuroblastoma.

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