The Phosphatidylinositol 3-Kinase/AKT Signal Transduction Pathway Plays a Critical Role in the Expression of p21WAF1/CIP1/SDI1 Induced by Cisplatin and Paclitaxel

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Abstract
The cyclin-dependent kinase inhibitor p21WAF1/CIP1/SDI1 (p21) plays a crucial role in DNA repair, cell differentiation, and apoptosis through regulation of the cell cycle. A2780 human ovarian carcinoma cells, which are sensitive to cisplatin and paclitaxel, express wild-type p53 and exhibit a p53-mediated increase in p21 in response to the chemotherapeutic agents. Here, we demonstrate that phosphatidylinositol 3-kinase (PI3K) and its downstream targets serine/threonine kinases AKT1 and AKT2 (AKT), are required for the full induction of p21 in A2780 cells treated with cisplatin or paclitaxel. Inactivation of the PI3K/AKT signal transduction pathway either by specific inhibitor LY294002 or by expression of dominant negative AKT inhibited p21 expression but had no inhibitory effect on the expression of the proapoptotic protein BAX by cisplatin and paclitaxel treatment. In addition, overexpression of wild-type or constitutively active AKT in A2780 cells sustained the regulation of p21 induction or increased the level of p21 expression, respectively. Experiments with additional ovarian carcinoma cell lines revealed that PI3K is involved in the expression of p21 induced by cisplatin or paclitaxel in OVCAR-10 cells, which have wild-type p53, but not in OVCAR-5 cells, which lack functional p53. These data indicate that the PI3K/AKT signal transduction pathway mediates p21 expression and suggest that this pathway contributes to cell cycle regulation promoted by p53 in response to drug-induced stress. However, inactivation of PI3K/AKT signaling did not result in significant alteration of the drug sensitivity of A2780 cells, suggesting that the cell death induced by cisplatin or paclitaxel proceeds independently of cell protective effects of PI3K and AKT.

Introduction
The antitumor agent cis-diaminedichloroplatinum(II) (cisplatin) is a DNA-reactive reagent widely used as a chemotherapeutic drug in the treatment of various human malignancies in combination with the antimicrotubule agent paclitaxel. Cisplatin treatment causes cell cycle arrest at various phases in the cell cycle and induces apoptosis (1). Paclitaxel stabilizes microtubules and disrupts the normal polymerization/depolymerization cycle of microtubules and is associated with the arrest of cells in the G2-M phase of the cell cycle (2). In human ovarian cancer cell lines, cisplatin or paclitaxel treatment at the clinically relevant concentration induces expression of the tumor suppressor protein p53, together with a coordinate increase in the cyclin/cyclin-dependent kinase inhibitor p21 protein, to maintain the correct temporal ordering of cell cycle events (3). However, whereas p53 is thought to induce p21 expression when DNA damage is caused by radiation or various chemotherapeutic drugs, recent investigations have demonstrated that transcriptional and posttranscriptional changes in p21 expression after DNA damage are not necessarily linked to p53 status (3–5). Various studies suggest that these processes are influenced by signal transduction pathways such as those that mediate cell growth, differentiation, and stress response (6, 7). For example, it has been reported that induction of p53 and p21 by paclitaxel requires Raf-1, an upstream regulator of the MAPK, in a human prostate cancer cell line (8). On the other hand, p53 and p21 were induced independently of Raf-1 in a paclitaxel-sensitive human lung carcinoma cell line (9).

PI3K and downstream AKT/protein kinase B family members have been implicated in disparate cell responses, including the protection of cells from apoptosis, the promotion of cell proliferation, and diverse metabolic responses (10). In fibroblasts, activated AKT is sufficient to protect cells from apoptosis induced by serum withdrawal (11). It has also been demonstrated that inactivation of PI3K activity markedly inhibits proliferation of small cell lung cancer cells by stimulating apoptosis and promoting cell cycle delay in G1 (12). Furthermore, PI3K/AKT-dependent signaling stimulates the induction of cyclin D1 expression by serum and regulates its repression by herbimycin A, a drug that binds to heat shock protein 90, at the level of mRNA translation (13). Thus, the PI3K/AKT signaling pathway seems to be involved in cell cycle regulation via ectopic stimulation factors. Of interest, it has been reported that the MEK1 inhibitor PD98059 partially inhibits PMA-induced p21 expression, whereas the PI3K inhibitor LY294002 had no such effect in the breast cancer cell line MCF-7 (14). Here, we demonstrate a direct link between the PI3K/AKT signaling pathway, but not the MEK/ERK signaling pathway, and p21 expression in the human ovarian carcinoma cell line A2780. Furthermore, the cell death induced by cisplatin or paclitaxel seems to occur independently of these signal transduction pathways and p21 expression.

Materials and Methods
Reagents. Cisplatin and paclitaxel were obtained from Bristol-Myers Squibb (Syracuse, NY). LY294002 and PD98059 were from Calbiochem (La Jolla, CA). Antiphosphorylated ERK mAb and LipofectAMINE were from Life Technologies, Inc. (Grand Island, NY). Anti-Bax, -p21, and -p53 mAbs were obtained from Calbiochem. Anti-HA mAb (HA.11) was from Babco (Richmond, CA). Anti-Akt polyclonal antibody was from Upstate Biotechnology (Lake Placid, NY). [α-32P]dATP was obtained from DuPont (New England Nuclear, Wilmington, DE).

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3 The abbreviations used are: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MEK1, MAPK kinase 1; PI3K, phosphatidylinositol 3-kinase; PMA, phorbol 12-myristate 13-acetate; mAb, monoclonal antibody; HA, hemagglutinin.
REGULATION OF P21 EXPRESSION BY PI3K/AKT

Cell Culture, Drug Treatment, and Preparation of Cell Extract. A2780, OVCAR-5, and OVCAR-10 cells were maintained in monolayer culture as described elsewhere (15). Cells (2 × 10^6)/well were plated in 6-well plates and grown overnight in RPMI 1640 containing 10% fetal bovine serum. In Fig. 1, porcine insulin (0.25 units/ml) was supplemented in the cell culture medium, as indicated in the figure legend. A2780 cells were incubated with culture medium containing 10 μM cisplatin or 50 nM paclitaxel for 24 h. OVCAR-5 and OVCAR-10 cells were incubated with culture medium containing 10 μM cisplatin or 100 nM paclitaxel for 24 h. We verified that in A2780 cells cisplatin or paclitaxel induces p53 and p21 in a dose- and time-dependent manner (data not shown). To inactivate PI3K or MEK1, 10 μM of LY294002 or PD98059, respectively, were added to the medium 1 h before cisplatin or paclitaxel treatment as indicated in the figure legends. We also confirmed that 10 μM of LY294002 or PD98059 is sufficient for specific inactivation of PI3K/AKT- or MAPK-mediated signaling, respectively, in A2780 cells (Fig. 1B and data not shown). Whole cell extracts were then prepared as described previously (16).

Immunoblot Analysis. Equal amounts of protein were subjected to 8% or 12.5% SDS-PAGE and transferred to Hybond-C membrane (Amersham, Arlington Heights, IL). Western blot analyses were carried out by using the appropriate antibody as noted in the figure legends, and protein bands were visualized with appropriate horseradish peroxidase-conjugated anti-IgG antibodies and enhanced chemiluminescence according to the manufacturer's recommendations (Amersham).

cDNA Clones and DNA Construction. Plasmid DNA constructs for the expression of HA-epitope-tagged forms of AKT (i.e., HA-Akt1, HA-AKT2, myrHA-Akt1, myrHA-AKT2, and HA-AKT2E299K) were prepared as described previously (16). The inactive Akt1 mutant HA-Akt1AA (threonine 308 and serine 473 replaced with alanines) was a gift from A. Bellacosa (Fox Chase Cancer Center, Philadelphia, PA). Transfections were carried out with 2 μg of plasmid DNA/2 × 10^5 cells, using LipofectAMINE reagent. Note that the related human AKT proteins are designated Akt1 and Akt2, whereas the murine protein homologous to human Akt1 is designated Akt1.

Northern Blot Analysis. A2780 cells (5 × 10^5) were cultured in 150-mm dishes, followed by treatment with LY294002 dissolved in DMSO and chemotherapeutic agents, as described above. Total cellular RNA was prepared by the guanidium isothiocyanate and phenol/chloroform extraction procedure. RNA samples (25 μg) were separated through 1% agarose gel and transferred onto nylon membranes (Gene Screen; DuPont). Membranes were hybridized with [α-32P]dATP-labeled p53 or p21 cDNA probes.

Cytotoxicity Assay. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was used to determine the relative sensitivities of A2780 cells to cisplatin and paclitaxel (17). Cells were plated in 150 μl of medium per well in 96-well plates (Corning Glass, Corning, NY). Following overnight incubation, LY294002 and PD98059 were added to a final concentration 10 μM each, and the plates were incubated for 1 h. To determine the IC50, various concentrations of cisplatin or paclitaxel were then added in a 10 μl volume. After a 72-h incubation, 50 μl of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide were added per well. After 2 h at 37°C, the cells were lysed by adding 100 μl of 20% (w/v) SDS, 50% (v/v) NN-dimethylformamide, pH 4.7 and incubated overnight at 37°C. The absorbance at 595 nm was measured for each well using a Bio-Tek ELx800 microplate reader (Bio-Tek Instruments, Winooski, VT).

Results

PI3K-dependent p21 Expression in A2780 Cells. A2780 cells express wild-type p53. The apoptotic response of A2780 cells to treatment with cisplatin or paclitaxel, at concentrations close to those that inhibit cell growth by 50% (IC50), is modest, and a p53-mediated increase in p21 has been reported (18–20). We first investigated whether induction of p21 can account for the difference in response of A2780 cells treated with cisplatin or paclitaxel in the presence or absence of serum. On withdrawal of serum, p21 expression levels were slightly elevated in untreated cells (Fig. 1A, compare Lanes 1 and 4), however, the induction of p21 in response to cisplatin or paclitaxel was abrogated (Fig. 1A, Lanes 5 and 6). When A2780 cells were cultured in the presence of insulin prior to serum starvation, they were still capable of inducing p21 in response to cisplatin or paclitaxel treatment (Fig. 1A, Lanes 7–9). These data suggest that the signaling activity in response to mitogenic stimulation is required for maximal p21 induction. To determine the relevant signal transduction pathway, we used inhibitors specific for the pathway (i.e., the MEK1 inhibitor PD98059 and the PI3K inhibitor LY294002). An anti-phospho ERK antibody that recognizes the dual phosphorylated active forms of p44/ERK1 and p42/ERK2 was used to analyze MAPK. As shown in Fig. 1B, preinactivation of PI3K by LY294002 partially inhibited p21 induction by cisplatin or paclitaxel treatment (Lanes 3 and 7). On the other hand, treatment with either cisplatin or paclitaxel activated the MAPK signal transduction pathway (Fig. 1B, Lanes 1, 2, 5, and 6), and the phosphorylation of ERK was specifically inhibited by PD98059 (Fig. 1B, Lanes 4 and 8). Preinactivation of the MAPK signal transduction pathway by PD98059, however, had no effect on p21 induction by either drug. These data indicate that PI3K, but not MAPK, participates in regulating p21 expression in response to cisplatin or paclitaxel treatment in A2780 cells.

Because previous studies have shown that in some instances the BAX gene is also transactivated by p53 (21, 22), we assessed the...
expression of BAX and p53 in cells treated with cisplatin or paclitaxel. As shown in Fig. 1B, PD98059 or LY294002 had little or no effect on the expression of BAX or p53 in response to cisplatin or paclitaxel treatment. Marked up-regulation of BAX expression by paclitaxel treatment was not observed in this experiment, presumably due to differences in the mechanism of cytotoxicity of cisplatin and paclitaxel (20). Overall, these data indicate that PI3K participates in the regulation of p21 expression, but it is not required for BAX expression.

To determine whether the responses seen in A2780 cells are generally applicable, similar experiments were performed with other human ovarian carcinoma cell lines. Inhibition of p21 induction by the inactivation of PI3K was observed in OVCAR-10 cells, which express wild-type p53, whereas no significant alteration of p21 expression was observed in OVCAR-5 cells, which lack p53 expression (Fig. 1C). Notably, although there is significant induction of p53 by cisplatin or paclitaxel treatment, a slight reduction in p53 expression was observed in both A2780 and OVCAR-10 cells treated with LY294002 (Fig. 1, B and C). These data suggest that PI3K plays an important role in p21 expression in cells expressing functional p53.

We next addressed whether PI3K is involved in p21 expression at the transcriptional level. Northern blot analyses were performed using p21 and p53 cDNAs as probes. As shown in Fig. 1D, although neither cisplatin nor paclitaxel treatment altered the level of p53 mRNA, they did induce an increase in p21 mRNA expression, which was partially suppressed by LY294002 treatment. Specifically, densitometric analysis revealed that LY294002 treatment reduced p21 mRNA induction by cisplatin and paclitaxel ~70% and 45%, respectively. Collectively, our findings suggest that PI3K is required for full up-regulation of p53 protein expression and plays a role in p21 induction at the transcriptional level.

**AKT Participates in Regulating p21 Expression.** We previously demonstrated that on insulin stimulation A2780 cells activate the serine/threonine kinases AKT1 and AKT2 in a PI3K-dependent manner (16). To assess the effect of AKT on p21 expression, HA-epitope-tagged wild-type Akt1 (HA-Akt1) or constitutively active Akt1 (myrHA-Akt1) was expressed in serum-deprived A2780 cells. Cells transfected with plasmid vector alone failed to induce p21 due to serum starvation, whereas overexpression of HA-Akt1 sustained the drug-dependent p21 induction (see pcDNA3 and HA-Akt1 in Fig. 2A). Furthermore, cells transfected with myrHA-Akt1 showed elevated levels of p21 expression (compare HA-Akt1 and myrHA-Akt1). Parallel experiments with AKT2 constructs showed similar results.

**Specific Involvement of AKT in p21 Expression.** To determine whether endogenous AKT contributes to p21 expression, we used catalytically inactive Akt1 and AKT2 expression constructs, HA-Akt1AA and HA-AKT2E299K, respectively (16). As expected, coexpression of dominant negative AKT mutants in A2780 cells, in the presence of serum, resulted in decreased levels of p21 expression compared with control cells and had no significant effect on the up-regulation of p53 expression (Fig. 2B, compare Lanes 1, 2, and 3 and Lanes 4, 5, and 6). Thus, the expression of dominant negative AKT mutants mimicked the effect of the PI3K inhibitor. Taken collectively, these data indicate that both PI3K and AKT play a critical role in regulating the expression of p21 in response to cisplatin and paclitaxel treatment in A2780 cells.

**Inactivation of PI3K/AKT Signaling Does Not Alter the Drug Sensitivity of A2780 Cells.** We next addressed whether abrogation of p21 induction via inactivation of PI3K affects the sensitivity of A2780 cells to cisplatin or paclitaxel treatment. A2780 cells were incubated with LY294002 before cisplatin or paclitaxel treatment. PD98059 was used for the comparative control. As shown in Table 1, neither LY294002 nor PD98059 affected the sensitivity of A2780 cells to cisplatin and paclitaxel. We also confirmed that there was no difference in the chemosensitivity of A2780 cells cultured in the presence or absence of insulin (data not shown). In addition, A2780 cells stably expressing HA-Akt1 or HA-AKT2 did not alter the drug sensitivity to cisplatin or paclitaxel (data not shown).

**Discussion**

Previous studies have demonstrated that in cells treated with cisplatin or paclitaxel, p53 promotes both cell cycle arrest and apoptosis, which is associated with the up-regulation of p21 and BAX expression, respectively (Fig. 3). Although treatment with either cisplatin or paclitaxel results in increased p53 protein levels in A2780 cells, transcriptional levels of p53 are not altered. Thus, the increases in p53 by these agents is likely to occur by posttranscriptional regulation, possibly due to enhanced protein stability (23). Our data demonstrate that either inactivation of PI3K or expression of dominant negative AKT inhibits p21 expression without significant effect on the regulation of BAX expression in A2780 cells (Fig. 3). In addition, expression of constitutively active or wild-type AKT enhanced p21 expression and sustained p21 induction in response to drug treatment.

### Table 1 Effect of MEK1 and PI3K inhibitors on cisplatin and paclitaxel sensitivity in A2780 cells

<table>
<thead>
<tr>
<th>Drug</th>
<th>Inhibitors</th>
<th>Cisplatin IC_{50}^{a}</th>
<th>Paclitaxel IC_{50}^{a}</th>
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<tbody>
<tr>
<td>None</td>
<td>PD98059</td>
<td>LY294002</td>
<td></td>
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<tr>
<td>0.32 ± 0.03</td>
<td>0.49 ± 0.13</td>
<td>0.44 ± 0.06</td>
<td></td>
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<tr>
<td>2.1 ± 0.1</td>
<td>2.0 ± 0.1</td>
<td>2.3 ± 0.2</td>
<td></td>
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</tbody>
</table>

^{a} Added at 10 μM 1 h before the drug treatment.

Unit in μM.

^{c} The IC_{50} value and the estimate of SD of the experiment mean for cisplatin and paclitaxel were determined in triplicate in two separate experiments.
Many oncogene and tumor suppressor gene products are components of signal transduction pathways that control cell cycle entry/exit, cell differentiation, DNA repair, cell survival/death, or stress response programs. Tumor promoter PMA induces p21 expression via ERK signaling in MCF-7 cells (14), and we also confirmed that in A2780 cells p21 is induced by PMA treatment in a MAPK-dependent manner without up-regulation of p53 expression (data not shown). Thus, these data suggest that cells use alternative independent signal transduction pathways for p21 induction and that the involvement of PI3K/AKT in p21 induction is specific to the stress induced by cisplatin or paclitaxel.

We previously demonstrated that A2780 cells are sensitive to cisplatin or paclitaxel treatment (15). However, little is known about the mechanism of cell death induced by cisplatin or paclitaxel. In the process of apoptosis, the release of cytochrome c into the cytoplasm is required for the activation of procaspase-3 by caspase-9 (31). It has also been demonstrated that Akt1 can phosphorylate and inactivate caspase-9 and suppress caspase-9-induced apoptosis in 293 cells (32). However, the cell death induced by cisplatin has been shown to proceed in a caspase-3-independent manner in A2780 cells (33). In addition, it has also been demonstrated that p21 is not required for p53-dependent apoptosis because thymocytes from transgenic mice lacking p21 genes are still capable of undergoing apoptosis after irradiation (34). Our studies provide additional support for these notions because alteration of p21 expression in A2780 cells by inactivation of the PI3K/AKT signal transduction pathway did not affect the sensitivity of cells in response to cisplatin or paclitaxel treatment. These data suggest that although p21 induction may be required to regulate the cell cycle as an emergency signal when cells are exposed to chemotherapeutic drugs, the cell death programs (including BAX expression induced by the drug) proceed independently from the process of cell cycle control (Fig. 3). Thus, future studies of the high drug sensitivity of A2780 cells may provide us with novel insights into mechanisms of drug sensitivity with respect to chemotherapy for the drug-resistant tumor cells.

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References


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