Akt-Mediated Cisplatin Resistance in Ovarian Cancer: Modulation of p53 Action on Caspase-Dependent Mitochondrial Death Pathway

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Abstract
Akt is a determinant of cisplatin [cis-diammine-dichloroplatinum (CDDP)] resistance in ovarian cancer cells, and this may be related to the regulation of p53. Precisely how Akt facilitates CDDP resistance and interacts with p53 is unclear. Apoptotic stimuli induce second mitochondria-derived activator of caspase (Smac) release from mitochondria into the cytosol, where it attenuates inhibitor of apoptosis protein–mediated caspase inhibition. Whereas Smac release is regulated by p53 via the transactivation of proapoptotic Bcl-2 family members, it is unclear whether p53 also facilitates Smac release via its direct mitochondrial activity. Here we show that CDDP induces mitochondrial p53 accumulation, the mitochondrial release of Smac, cytochrome c, and HTR/Omi, and apoptosis in chemosensitive but not in resistant ovarian cancer cells. Smac release was p53 dependent and was required for CDDP-induced apoptosis. Mitochondrial p53 directly induced Smac release. Akt attenuated mitochondrial p53 accumulation and Smac/cytochrome c/Omi release and conferred resistance. Inhibition of Akt facilitated Smac release and sensitized chemoresistant cells to CDDP in a p53-dependent manner. These results suggest that Akt confers resistance, in part, by modulating the direction of action of p53 on the caspase-dependent mitochondrial death pathway. Understanding the precise etiology of chemoresistance may improve treatment for ovarian cancer.

Introduction
Cisplatin-centred chemotherapy is an important treatment modality for human ovarian cancer. However, the development of chemoresistance is a major hurdle limiting treatment success. Whereas the precise molecular mechanisms of chemoresistance are poorly understood, recent evidence suggests that the failure of drug-induced apoptosis may be an underlying factor. We and others have identified a number of key mediators of apoptosis, the regulation of which is altered in chemoresistant cells (1–3).

Second mitochondria-derived activator of caspases (Smac), also known as direct inhibitor of apoptosis protein (IAP) binding protein with low isoelectric point (DIABLO), is a mitochondrial protein containing an NH2-terminal 55-amino-acid mitochondrial import sequence, which is released from mitochondria into the cytosol in response to apoptotic stimuli (4, 5). Once released into the cytosol, Smac docks to IAPs within the baculovirus IAP repeat domains via an NH2-terminal motif, thereby eliminating the inhibitory effects of IAPs on caspase-3, caspase-7, and caspase-9 (6). In addition, the interaction of Smac with IAPs results in a rapid ubiquitination and subsequent degradation of released Smac, which is mediated by the ubiquitin-protein ligase (E3) function of some IAPs (4, 7, 8). Recent studies have shown that mitochondrial Smac release is suppressed by Akt, Bcl-2, and Bcl-XL, but promoted by Bax, Bad, and Bid (4, 7–10).

TP53 is the most frequently mutated gene in human cancer (11). The TP53 gene product, p53, is a key regulator of the cellular response to cell stress and is rapidly up-regulated in response to DNA-damaging agents such as cisplatin (12, 13). p53 levels are primarily controlled by the proto-oncoprotein mouse double minute 2 (MDM2), which ubiquitinates p53 and facilitates its proteasomal degradation (14–16). Stabilization and activation of p53 following DNA damage occur through its site-specific phosphorylation, which attenuates the binding of MDM2 to p53 and facilitates the interaction of p53 with coactivators, such as p300, which increase affinity of p53 for consensus DNA sequences within the promoter region of p53-responsive genes (15, 17–19).

Akt/protein kinase B is a major downstream effector of phosphotyrosylinositol 3-OH-kinase–regulated serine/threonine kinase (20, 21). Whereas Akt is normally activated by growth factors in a phosphotyrosylinositol 3-OH-kinase–dependent manner, both phosphotyrosylinositol 3-OH-kinase and Akt are frequently activated and/or overexpressed in ovarian cancer (2, 3). Akt promotes cell survival, suppresses apoptosis, and regulates cis-diammine-dichloroplatinum (CDDP) sensitivity in ovarian cancer cells (1–3).

We previously showed that Akt is an important regulator of both X-linked IAP (XIAP) and p53 levels after CDDP challenge and that the functional p53 status is a determinant of Akt-mediated chemoresistance (2, 3). p53 induces apoptosis and regulates cytochrome c release by modulating the transcription of proapoptotic members of the Bcl-2 family (e.g., Bax and PUMA; refs. 10, 22) and by a transcription-independent mechanism involving direct translocation of p53 protein to mitochondria followed by inhibitory interactions with Bcl-2 and Bcl-XL (23). Whereas nuclear p53 can increase mitochondrial Smac release via the transcriptional pathway by inducing PUMA, Noxa, and Bax expression (10, 22, 24, 25), it remains unclear whether mitochondrial p53 can also directly trigger Smac release from mitochondria. Moreover, whereas Akt regulates p53 action, preserves mitochondrial integrity, and prevents mitochondrial Smac release (8, 9), it is not known whether these processes interact directly at mitochondrial level to regulate chemosensitivity.

In the current study, we show that CDDP-induced mitochondrial Smac release is a determinant of chemosensitivity in ovarian cancer cells. Moreover, we show that p53 can directly facilitate Smac release at the level of mitochondria and that Akt can inhibit
the mitochondrial release of proapoptotic proteins, including Smac, cytochrome c, and HtrA2/Omi, which facilitate caspase-dependent apoptosis, in part, by blocking mitochondrial p53 accumulation. The results suggest that modulation of these key cell fate regulators may be an effective means of overcoming chemoresistance in human ovarian cancer.

Materials and Methods

Reagents. Cells were cultured at 37°C with 5% CO2 in RPMI 1640 (OV2008 and C13*) or DMEM/F-12 (A2780s, A2780cp). Medium was supplemented with 10% fetal bovine serum, streptomycin (100 μg/mL), penicillin (100 units/mL), and fungizone (0.625 μg/mL). CDDP, DMSO, digitonin, Rotenon, phenylmethylsulfonyl fluoride (PMSF), and Hoechst 333258 were supplied by Sigma (Oakville, Ontario, Canada). The Smac-N7 peptide (AVPIAQKPRQIKWFRMKWKK) was modified to be cell permeable by linking the COOH-terminal lysine to the arginine of Antennapedia homeodomain 16-mer peptide via a proline linker, which was purchased from Calbiochem, Inc. (San Diego, CA) and its control peptide (MKSDFYFPRQQ-KWFWQNRMKWKK; ref. 10) was synthesized by Dr. Ajoy Basak (Ottawa Health Research Institute, University of Ottawa, Ottawa, Ontario, Canada). MG132 was supplied by Calbiochem. Smac siRNA and p53 siRNA were from Santa Cruz Biotechnology (Santa Cruz, CA) and Cell Signaling, Inc. (Danvers, MA) respectively. Negative control siRNA was from DHarmaco, Inc. (Chicago, IL). Ribojuice siRNA transfection reagent was from Novagen (Madison, WI). Adenoviral LacZ adenovirus was used to normalize the total concentration of adenovirus adenoviral constructs as indicated in the text. Infection with LacZ adenovirus was preincubated with recombinant wild-type p53 or bovine serum albumin (BSA; control) for 30 minutes at 37°C in 200 μL KCI buffer (15 mmol/L HEPES/NaOH, 125 mmol/L KCl, 4 mmol/L MgCl2, 5 mmol/L Na3HPO4, 0.5 mmol/L EGTA, 5 μmol/L Rotenon, 5 mmol/L succinate (pH 7.4)), then centrifuged at 13,000 × g for 10 minutes at 4°C. Mitochondrial pellets (5 μg) and corresponding supernatants were immunoblotted for p53 and Smac.

Western blot analyses. Western blotting was done as previously described (3). Membranes were incubated overnight at 4°C in primary antibodies (anti-Smac, 1:500; anti-actin, 1:2,000; anti-Cox-4, 1:1,000; anti-HA, 1:1,000; anti-LDH, 1:1,000; anti-p53, 1:1,000; anti-Bax, 1:1,000; anti-cytochrome c, 1:1,000; anti-HTR/Omi, 1:1,000), followed by horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibody (1:2,000) incubation at room temperature for 1 hour. Peroxidase activity was visualized with enhanced chemiluminescent kit (Amersham Biosciences, Piscataway, NJ). Results were scanned and analyzed using Scion Image software (Scion, Inc., Frederick, MD).

Results

CCDP induces mitochondrial Smac release and apoptosis in chemosensitive, but not in chemoresistant, ovarian cancer cells. To determine the relationship between subcellular Smac distribution and chemosensitivity, chemosensitive ovarian cancer cells (OV2008 and A2780s) and their resistant variants (C13* and A2780cp) were cultured with 10 μmol/L CDDP (DMSO as control) for different durations (0-24 hours). Mitochondrial and cytosolic Smac levels were determined by Western blotting. As shown in Fig. 1A and B, exposure of OV2008 or A2780s cells to CDDP decreased mitochondrial Smac and increased cytosolic Smac levels in a time-dependent fashion but had no effect on mitochondrial or cytosolic Smac levels in C13* or A2780cp cells. This was also associated with the release of the proapoptotic mitochondrial RNA interference. OV2008 cells were transfected with 0 to 100 nmol/L Smac or negative control siRNA for 48 hours whereas C13*-DNAkt2 cells were transfected with 50 nmol/L p53 or negative control siRNA for 24 hours as previously reported (27). Cells were then treated with CDDP and harvested for subsequent analysis as indicated.

Preparation of whole-cell lysates and subcellular fractions. Cells were collected after different treatments and preparation of whole-cell lysates was done as previously described (3). Subcellular cell fractions were prepared by digitonin-based permeabilization buffer as described by Gao et al. (28). The relative purity of the subcellular fractions was confirmed by Western blot using anti-LDH (cytosolic marker), anti-c23 (nuclear marker), and anti-Cox-4 (mitochondrial marker) antibodies.

In vitro mitochondrial Smac release. Purified mitochondrial fractionations were done as described by Yang et al. (29) and Marchenko et al. (30). Briefly, cell pellets were resuspended with buffer A [20 mmol/L HEPES-KOH (pH 7.5), 10 mmol/L KCl, 1.5 mmol/L MgCl2, 1 mmol/L sodium EDTA, 1 mmol/L sodium EGTA, 1 mmol/L DTT, and 100 mmol/L PMSF] containing 250 mmol/L sucrose. The cells were homogenized with 26-gauge needles and centrifuged twice at 750 × g for 10 minutes at 4°C. The supernatants were centrifuged at 10,000 × g for 15 minutes at 4°C, and the resulting mitochondrial pellets were layered over a 1 to 2 mol/L sucrose step gradient [10 mmol/L Tris (pH 7.6), 5 mmol/L EDTA, 2 mmol/L DTT, protease inhibitors] and centrifuged at 4°C for 30 minutes at 22,000 × g. Mitochondria were collected at the 1 to 1.5 mol/L interphase.

As previously reported by Mihara et al. (23), mitochondria (70 μg protein) were incubated with recombinant wild-type p53 or bovine serum albumin (BSA; control) for 30 minutes at 30°C in 200 μL KCl buffer [15 mmol/L HEPES/NaOH, 125 mmol/L KCl, 4 mmol/L MgCl2, 5 mmol/L Na3HPO4, 0.5 mmol/L EGTA, 5 μmol/L Rotenon, 5 mmol/L succinate (pH 7.4)], then centrifuged at 13,000 × g for 10 minutes at 4°C. Mitochondrial pellets (5 μg) and corresponding supernatants were immunoblotted for p53 and Smac.

proteins cytochrome c and HTR/Omi in chemosensitive OV2008 cells but not in resistant C13* cells. Furthermore, the accumulation of these proteins in the cytoplasm of chemosensitive cells was associated with an increase in the percentage of cells undergoing apoptosis. However, CDDP failed to induce apoptosis in C13* or A2780cp cells during these culture durations. In addition, these responses of the chemosensitive cells, but not of their resistant variants, were concentration dependent. Taken together, these data show that CDDP induces mitochondrial Smac/cytochrome c/HtrA2/Omi release and apoptosis in chemosensitive, but not in chemoresistant, ovarian cancer cells.

**Smac is required for CDDP-induced apoptosis.** Smac is rapidly degraded by the proteasome following its release from the mitochondria (7). To assess the possible confounding effects of proteasomal degradation of Smac in the present experiments, OV2008 cells were pretreated with or without the proteasome inhibitor MG132 (250 nmol/L) and then exposed to CDDP for 24 hours. Western blotting revealed that treatment with MG132 increased cytosolic Smac content irrespective of the presence of CDDP but did not markedly affect mitochondrial Smac content (Fig. 2A), suggesting that proteasomal degradation is also an important mechanism by which cytoplasmic Smac content is regulated. CDDP-induced apoptosis was assessed by Hoechst staining. A, OV2008 and C13* cells; B, A2780s and A2780cp cells.
To determine whether Smac is required for CDDP-induced apoptosis, OV2008 cells were transfected with Smac siRNA (0-100 nmol/L; 48 hours) and then treated with CDDP (10 μmol/L; 24 hours). Smac down-regulation was confirmed by Western blotting. Down-regulation of Smac significantly attenuated CDDP-induced apoptosis in these cells (Fig. 2B), suggesting that Smac is required for efficient CDDP-induced apoptosis.

Recent studies have shown that Smac inhibits XIAP by binding to its baculovirus IAP repeat domains, thereby interfering with its inhibitory effects on caspases (32). We previously showed that down-regulation of XIAP enhances CDDP sensitivity in chemoresistant cells (31). In addition, overexpression of Smac or addition of an N\textsubscript{H\textsubscript{2}}-terminal Smac heptapeptide (Smac-N7) significantly increased TRAIL-induced apoptosis in TRAIL-resistant Bax and Bak double-knockout mouse embryonic fibroblast cells (10). To determine whether an increasing Smac activity is sufficient to sensitize resistant cells to CDDP-induced apoptosis, C13* cells were pretreated with Smac-N7 peptide (20 μmol/L; 3 hours), followed by CDDP treatment for 24 hours. SMAC peptide alone had little effect on apoptosis, it markedly sensitized the cells to CDDP-induced apoptosis in a concentration-dependent manner (Fig. 2C). MG132 further enhanced the apoptotic response of C13* cells to CDDP-induced apoptosis (**, P < 0.01). These results show that Smac is an important mediator of CDDP-induced apoptosis and that increasing Smac activity is an effective means of overcoming chemoresistance in ovarian cancer cells.

CDDP induces mitochondrial p53 accumulation in chemosensitive, but not in chemoresistant, ovarian cancer cells. p53 induces apoptosis by target gene regulation and transcription-independent signaling (23, 33, 34). Recent studies have shown that p53 has a direct apoptogenic role at mitochondria (23). However, it is unclear whether p53 accumulates at the mitochondria following CDDP challenge in ovarian cancer cells. To determine the relationship between mitochondrial p53 accumulation and CDDP sensitivity in ovarian cancer cells, OV2008, C13*, A2780s, and A2780cp cells were treated with CDDP (0-10 μmol/L; 24 hours). Mitochondrial fractions and whole-cell lysates were analyzed by Western blot. C13* and A2780cp cells expressed higher whole-cell p53 levels in the absence of CDDP compared with OV2008 and A2780s. However, whole-cell p53 content in the chemosensitive cells was up-regulated by CDDP whereas that in the resistant cells was largely invariant. Furthermore, whereas p53 also accumulated in mitochondria of chemosensitive cells in response to CDDP, no accumulation was observed in the resistant cells despite similar whole-cell p53 levels between cell types (Fig. 3). These results suggest that the specific mitochondrial accumulation of p53 may not simply be secondary to its presence in the whole cell.

p53 regulates cytochrome c and Smac release via gene transactivation, such as up-regulation of Bax and PUMA (34, 35).
Mitochondrial p53 interacts with and inhibits Bcl-XL and promotes cytochrome c release by inducing Bak/Bax oligomerization and outer mitochondrial membrane permeabilization (23, 36). However, it is unclear whether p53 directly affects mitochondrial Smac release. We therefore asked whether the differential ability of mitochondria from chemosensitive and chemoresistant cells to accumulate p53 and release Smac is due to intrinsic differences in the mitochondria of these cells. To this end, mitochondria isolated from OV2008 and C13* cells were incubated with recombinant wt p53 (0-400 nmol/L) or with BSA as control. p53 accumulated at mitochondria and triggered Smac release equally well in both cell types. This effect occurred in a concentration-dependent manner with a maximum release with 200 nmol/L wt p53 within 30 minutes (Fig. 4A). These results show that p53 can directly induce mitochondrial Smac release and suggest that the observed differences in mitochondrial p53 accumulation and Smac release between chemosensitive and chemoresistant cells in response to CDDP are not at the mitochondrial level but result from a pre-mitochondrial failure to up-regulate/activate p53 in chemoresistant cells.

To determine whether this ability of p53 to directly induce Smac release also occurs in living cells, OV2008 and C13* cells were transfected with Mito p53, and WT p53, Mito c-Rel, ER p53, and ectromelia virus (EV) used as controls, followed by MG132 treatment (25 μmol/L; 3 or 10 hours). Mito p53 increased mitochondrial p53 content and induced a decrease in mitochondrial Smac content and an increase in cytosolic Smac content in both CDDP-sensitive and CDDP-resistant ovarian cancer cells after 3 hours of MG132 treatment, whereas WT p53 increased mitochondrial p53 content and induced a decrease in mitochondrial Smac content and an increase in cytosolic Smac content in both CDDP-sensitive and CDDP-resistant ovarian cancer cells after 3 hours of MG132 treatment, whereas WT p53 increased nuclear p53 content but failed to induce Smac release until 10 hours. Bax up-regulation was not observed until 10 hours posttreatment. Whereas there was a significant difference among transfection groups (\( P < 0.05 \)), there was no significant difference between the two cell lines (\( P > 0.05 \)).
empty vector were used as controls. Mito p53 directly triggered Smac release and apoptosis in both CDDP-sensitive and CDDP-resistant cells after 3 hours. By contrast, WT p53 increased nuclear p53 content at both time points but did not induce Smac release or apoptosis until 10 hours. To assess p53 transactivation activity, Bax content was monitored by Western blot. Bax content increased only after 10 hours, suggesting that p53-mediated Smac release and apoptosis after 3 hours were independent of p53 transactivation activity (Fig. 4B). Taken together, these findings show that CDDP induces mitochondrial p53 accumulation in chemosensitive, but not in resistant, cells, and suggest that this does not arise from any intrinsic differences in properties between mitochondria of both cell types. Importantly, these results show that p53 directly induces mitochondrial Smac release.

**Akt inhibits CDDP-induced mitochondrial Smac release and apoptosis in ovarian cancer cells.** Akt is a determinant of chemoresistance in ovarian cancer cells (3). Although Smac and cytochrome c release is regulated by Akt in PC12 cells (8), and how Akt regulates CDDP-induced Smac release is not known nor is the involvement of this process in the regulation of chemosensitivity. To examine the effect of Akt on CDDP-induced Smac release, chemosensitive wt-p53 ovarian cancer cells (A2780s-PMH6), stably transfected with constitutively active Akt2 (A2780s-AAkt2), were treated with CDDP (0-10 μmol/L; 24 hours). Compared with A2780s-PMH6 cells, A2780s-AAkt2 cells showed a significant suppression of CDDP-induced mitochondrial Smac, HtrA2/Omi, and cytochrome c release. Even in the absence of CDDP, basal cytosolic levels of these mitochondrial proteins in A2780s-PMH6 cells were higher than those in A2780s-AAkt2 cells, suggesting that Akt activation suppresses both basal and CDDP-induced Smac release (Fig. 5A). Constitutively activated Akt2 also reduced the sensitivity of A2780s cells toward CDDP (Fig. 5A), an effect that is consistent with our previous results (3).

To further examine the role of Akt2 in the regulation of Smac release in CDDP-induced apoptosis, we extended these observations with a concentration-response study using C13* (chemoresistant, wt p53) and C13*ΔNAkt2 cells (C13* cells stably transfected with DN-Akt2). Western blot analyses showed that whereas CDDP failed to reduce mitochondrial Smac, increase cytosolic Smac, or induce apoptosis in C13* cells, these effects were facilitated by expression of DN-Akt2 (Fig. 5B). These findings suggest that Akt2 regulates CDDP-induced Smac release and is a determinant of chemoresistance in ovarian cancer cells.

To ascertain whether Akt1 is also involved in Smac release, C13* cells were infected with adenoviral DN-Akt1 or LacZ (MOI, 0-80; 24 hours). HA-tagged DN-Akt1 construct was detected by Western blot using anti-HA antibody. After 24-hour infection, cells were incubated with CDDP (10 μmol/L; 24 hours). Western blot analyses showed decreased mitochondrial Smac content and increased cytosolic Smac content with increasing DN-Akt1 concentration in the presence of CDDP, although DN-Akt1 alone failed to increase mitochondrial Smac release (Fig. 5C). We extended the experiment to different concentrations of CDDP (0-10 μmol/L) after DN-Akt1 infection (MOI, 80). Expression of DN-Akt1 alone had a minimal effect on mitochondrial Smac release. However, in the presence of CDDP, Smac content was decreased in the mitochondria and increased in the cytosol in C13* cells infected with DN-Akt1. Infection with LacZ alone did not alter Smac contents in either cytosol or mitochondria (Fig. 5D). Furthermore, whereas down-regulation of Akt1 function alone did not significantly induce apoptosis, it sensitized C13* cells to CDDP-induced apoptosis (Fig. 5C and D). These findings suggest that both Akt1 and Akt2 are involved in the regulation of CDDP-induced mitochondrial Smac release and Akt-mediated chemoresistance.

**Akt suppresses p53 accumulation at mitochondria.** As shown, mitochondrial Smac release was directly triggered by p53 and inhibited by Akt. However, it is unclear whether Akt interferes with mitochondrial p53 accumulation. To examine this possibility, A2780s-PMH6 and A2780s-AAkt2 cells were treated with CDDP (0-10 μmol/L; 24 hours). In the absence of CDDP, A2780s-AAkt2 cells expressed more whole-cell p53 than control cells; however, CDDP increased whole-cell p53 content in A2780s-AAkt2, but less so in the A2780s-AAkt2 cells. In addition, whereas CDDP induced mitochondrial p53 accumulation in A2780s-PMH6, this effect was markedly reduced in A2780s-AAkt2 cells (Fig. 6A). These findings show that Akt inhibits CDDP-induced mitochondrial p53 accumulation. Interestingly, the failure of CDDP to induce mitochondrial p53 accumulation in the A2780s-AAkt2 cells could not be explained by the absence of p53 within the cell. Thus, Akt likely attenuates the specific translocation of p53 to the mitochondria. Furthermore, the results suggest that Akt may inhibit p53-dependent mitochondrial Smac release through this mechanism.

To further explore this hypothesis, C13* and C13*ΔNAkt2 cells were incubated with CDDP (0-10 μmol/L; 24 hours). p53 accumulated in the mitochondria after down-regulation of Akt2 in C13* cells even without CDDP treatment; however, this effect was markedly enhanced by CDDP (Fig. 6B).

Taken together, these data show that Akt blocks specific mitochondrial p53 accumulation and may, in part, confer chemoresistance via this mechanism.

**Akt inhibits CDDP-induced, p53-mediated mitochondrial Smac release.** We have shown that Akt is a determinant of CDDP-induced apoptosis and p53 function is required for sensitization to CDDP through suppression of Akt activity (3). To determine whether p53 is required for DN-Akt-mediated mitochondrial Smac release, A2780cp (p53-mutant chemoresistant cells) and A2780cp-DNAkt2 cells (stably transfected with DN-Akt2) were treated with CDDP (0-10 μmol/L; 24 hours). Down-regulation of Akt failed to facilitate Smac release or sensitize A2780cp cells to CDDP, suggesting that wt p53 is important in CDDP-induced mitochondrial Smac release and apoptosis (Fig. 6B, lane 1, and data not shown).

To determine whether p53 status is indeed a determinant of Akt-regulated mitochondrial Smac release, A2780cp-DNAkt2 cells were infected with adenoviral wt p53 (MOI, 0-20; 24 hours). Reconstitution of wt p53 increased Smac release and sensitized these cells to CDDP in the presence of DN-Akt2. This effect was dependent on the concentration of wt p53 (Fig. 7A). These findings further suggest that Akt attenuates mitochondrial Smac release in chemoresistant cells and that suppression of Akt function sensitizes chemoresistant cells to CDDP in a p53-dependent manner.

To further ascertain the role of p53 in promoting mitochondrial Smac release after down-regulation of Akt, C13*ΔN Akt2 (wt p53) cells were transfected with p53 siRNA (50 nmol/L; 24 hours), followed by CDDP treatment (0-20 μmol/L; 24 hours). Down-regulation of p53 markedly reduced mitochondrial Smac release, an effect that was associated with decreased apoptosis to CDDP (Fig. 6C), indicating that Akt-modulated mitochondrial Smac release is dependent on p53 function.

In the present studies, we have shown that Akt blocks CDDP-induced mitochondrial p53 accumulation and that Akt-regulated mitochondrial Smac release is dependent on p53 function. To further examine whether Akt prevents CDDP-induced, p53-dependent apoptosis...
mitochondrial Smac release, A2780cp and A2780cp-DN-Akt2 cells were infected with adenoviral wt p53 (MOI, 0-20; 24 hours), followed by 24-hour CDDP treatment. Whereas infection of wt p53 induced mitochondrial Smac release in both A2780cp and A2780cp-DN-Akt2 cells, down-regulation of Akt2 enhanced CDDP-induced mitochondrial Smac/Omi/cytochrome c release. Constitutively activated Akt2 also reduced the sensitivity of A2780 cells toward cisplatin-induced apoptosis (*, P < 0.05; ***, P < 0.001; B, C13* and C13*-DN-Akt2 (stable transfection with DN-Akt2) cells were treated with CDDP (0-10 μmol/L; 24 hours); mitochondrial Smac release increased in chemoresistant cells expressing DN-Akt2 in response to CDDP. Down-regulation of Akt2 sensitized C13* cells to CDDP (**, P < 0.01). C, C13* cells were infected with different MOIs of adenoviral DN-Akt1 (MOI, 0-80) and LacZ (Control, MOI, 80-0) for 24 hours, followed by CDDP (10 μmol/L; 24 hours; DMSO was used as a control), CDDP decreased mitochondrial Smac content and increased cytosolic Smac content after down-regulation of Akt1. Western blot (anti-HA) confirmed expression of DN-Akt1. D, C13* cells were infected with adenoviral DN-Akt1 and LacZ (MOI, 80; LacZ as control; 24 hours), followed by different concentrations of CDDP (0-10 μmol/L; 24 hours). Mitochondrial Smac content decreased and cytosolic Smac content increased after down-regulation of Akt1.

Discussion
In the present study, we have shown that CDDP-induced mitochondrial Smac, cytochrome c, and Omi release is a determinant of chemosensitivity in ovarian cancer cells. Moreover, our data show that p53 can directly induce mitochondrial Smac release and suggest that Akt promotes chemoresistance, in part, by modulating the direction action of p53 on the caspase-dependent mitochondrial death pathway. Finally, our data suggest that translocation of p53 to the mitochondria is an active process and show that Akt can specifically attenuate this process.

The development of chemoresistance is a major hurdle limiting treatment success for human ovarian cancer. However, the molecular mechanisms underlying chemoresistance are varied and poorly understood. Recent data suggest that dysregulation
of apoptosis is a key contributor to chemoresistance. Smac is released from mitochondria to the cytosol after apoptotic stimuli and binds to XIAP, c-IAP-1, or c-IAP-2, and abrogates IAP-mediated inhibition of caspase-3 and caspase-7, thereby facilitating caspase-mediated apoptosis (4, 37, 38). Whereas chemotherapeutic agents can induce Smac and cytochrome c release (39), if and how Smac plays a role in CDDP-induced apoptosis and whether dysregulation of Smac may be an etiologic factor in chemoresistance are unclear.

We previously showed that CDDP induces apoptosis in chemosensitive ovarian cancer cells but not in their resistant variants (2, 3, 31). We have extended these studies to investigate the role of Smac in CDDP-induced apoptosis in ovarian cancer cells. In the present study, we found that CDDP-induced mitochondrial Smac release was associated with chemosensitivity, suggesting that Smac release may be a determinant of CDDP-induced apoptosis. Furthermore, down-regulation of Smac by RNA interference could not completely suppress CDDP-induced apoptosis (40). However, recent reports have shown that p53 has a direct apoptogenic role at the mitochondria and directly triggers cytochrome c release by binding to Bcl-2, Bcl-XL (23). We have shown that CDDP induces mitochondrial p53 accumulation in chemosensitive cells but not in their resistant counterparts. In addition, total p53 levels were approximately equivalent in both cell types although the levels were largely invariant in the resistant cells in response to CDDP. This suggests that it is the specific mitochondrial accumulation of p53 that is disrupted in chemoresistant cells, rather than the absence of total p53 from these cells. This represents, to our knowledge, the first demonstration of a physiologic condition under which mitochondrial p53 accumulation is alternately regulated. Moreover, this disparity does not arise from intrinsic differences between mitochondria in these cells because recombinant wt p53 directly induced Smac release from isolated mitochondria of both cell types. Instead, this difference likely results from the influence of premitochondrial factors promoting p53 accumulation/activation, which may contribute to chemoresistance. Smac plays a role in CDDP-induced apoptosis and whether dysregulation of Smac may be an underlying cause of chemoresistance and that the failure to activate the caspase-dependent mitochondrial death pathway may contribute to chemoresistance.

p53 mediates apoptosis by transcriptional activation of proapoptotic genes, such as Bax, which facilitate apoptosis by promoting cytochrome c release (22, 34, 40). However, recent reports have shown that p53 has a direct apoptogenic role at the mitochondria and directly triggers cytochrome c release by binding to Bcl-2, Bcl-XL (23). We have shown that CDDP induces mitochondrial p53 accumulation in chemosensitive cells but not in their resistant counterparts. In addition, total p53 levels were approximately equivalent in both cell types although the levels were largely invariant in the resistant cells in response to CDDP. This suggests that it is the specific mitochondrial accumulation of p53 that is disrupted in chemoresistant cells, rather than the absence of total p53 from these cells. This represents, to our knowledge, the first demonstration of a physiologic condition under which mitochondrial p53 accumulation is alternately regulated. Moreover, this disparity does not arise from intrinsic differences between mitochondria in these cells because recombinant wt p53 directly induced Smac release from isolated mitochondria of both cell types. Instead, this difference likely results from the influence of premitochondrial factors promoting p53 accumulation/activation, which may be impaired in resistant cells. Although it has been shown that p53 induces Smac release through its transcriptional function (22, 34), the present findings represent the first demonstration that p53 can directly induce mitochondrial Smac release.
and suggest that pre-mitochondrial factors may regulate p53 mitochondrial accumulation and p53-dependent Smac release. Furthermore, the results suggest that failure of p53 to accumulate in the mitochondria of chemoresistant cells may underlie the inability of CDDP to induce mitochondrial Smac release in these cells. Interestingly, expression of mitochondrial-targeted p53 increases mitochondrial Smac release and apoptosis in both sensitive and resistant cells much faster (3 hours) than wt p53 (10 hours), suggesting that the mitochondrial effects of p53 may be critical during the early phase of apoptosis.

Mitochondrial Smac release is suppressed by Akt, Bcl-2, and Bcl-XL, but promoted by Bax, Bad, and Bid (8, 24, 41, 42). Akt inhibits Bid cleavage and Bax activation, thereby inhibiting cytochrome c and Smac release and suppressing apoptosis (8, 9). Here we showed that Akt activation attenuated CDDP-induced Smac (and cytochrome c/Omi) release and apoptosis whereas inhibition of Akt function facilitated these responses in wt-p53 chemoresistant cells. These results confirm that Akt is a determinant of CDDP resistance in ovarian cancer cells and suggest that regulation of Smac release may be one mechanism by which Akt confers chemoresistance.

Significantly, our data show that Akt activation inhibits mitochondrial p53 accumulation whereas inhibition of Akt function promotes CDDP-induced mitochondrial import of p53. This suggests that Akt may regulate Smac release and apoptosis by attenuating the mitochondrial actions of p53. Furthermore, whereas the mitochondrial accumulation of p53 in response to various cellular stimuli and the proapoptotic role of mitochondrial p53 have been shown (23, 43), the present report represents, to our knowledge, the first evidence of a cellular control mechanism governing this process. In particular, whereas Akt has been shown to affect nuclear p53 function (i.e., DNA binding/transactivation) and/or to alter p53 content by activating MDM2 (44–46), the current study provides strong evidence that Akt may serve a more wide-ranging antiapoptotic role by interfering with the mitochondrial accumulation of p53. Because mitochondrial p53 accumulation is correlated with p53-induced apoptosis and not cell cycle arrest (23), this strongly suggests that prevention of mitochondrial accumulation of p53 by Akt may be a central mechanism by which Akt interferes with the normal execution of apoptosis.

**Figure 7.** Akt inhibits CDDP-induced p53-dependent mitochondrial Smac release. **A,** A2780cp and A2780cp-DNAkt2 cells (mutant type p53) were infected with adenoviral p53 (MOI, 20) for 24 hours, followed by CDDP treatment (10 μmol/L; 24 hours). p53 induced mitochondrial Smac, Omi, and cytochrome c release in both A2780cp and A2780cp-DNAkt2 cells in the absence of CDDP, and CDDP enhanced p53-induced release of these proteins after down-regulation of Akt. The changes in Smac, Omi, and cytochrome c content in A2780cp and A2780cp-DNAkt2 were associated with their increased apoptotic response. **B,** A2780cp and A2780cp-DNAkt2 cells (mutant type p53) were infected with adenoviral p53 (MOI, 20; 24 hours), followed by CDDP treatment (10 μmol/L; 24 hours). Whereas cotreatment with CDDP and p53 (MOI, 20) induced mitochondrial Smac, Omi, and cytochrome c release in A2780cp, these effects were enhanced in A2780cp-DNAkt2 cells. The changes in Smac, Omi, and cytochrome c content in A2780cp and A2780cp-DNAkt2 were associated with their increased apoptotic response.
p53 functional status is a determinant of chemosensitivity in ovarian cancer cells (3). Moreover, suppression of Akt function sensitizes wt-p53, but not p53-mutant, chemoresistant cells to CDDP-induced apoptosis, suggesting that Akt-mediated chemoresistance may be critically dependent on suppression of p53 function. In the current study, we found that whereas down-regulation of Akt facilitated CDDP-induced mitochondrial Smac release in wt-p53 chemoresistant cells, this effect was not observed in p53-mutant chemoresistant cells, unless wt p53 was reconstituted to these cells, nor in wt-p53 cells where p53 expression was attenuated by RNA interference, suggesting that the effectiveness of DN-Akt as a means to facilitate Smac release and overcome chemoresistance is dependent on p53 function. These results suggest that p53 may mediate CDDP-induced mitochondrial Smac release and that Akt may block this release by interferring with a p53-dependent process.

As a whole, this work establishes that mitochondrial Smac release is an important contributor to CDDP-induced apoptosis and shows that chemoresistance is, in part, mediated through the ability of Akt to attenuate this p53-dependent process. Furthermore, Smac release can be triggered by the accumulation of p53 at the mitochondria where it directly induces its effects on Smac. Because mitochondrial p53 accumulation is attenuated in resistant cells in response to CDDP and is restored in these cells by inhibition of Akt, it seems likely that this event is a critical intermediary step in the process of CDDP-induced apoptosis.

Our data suggest that mitochondrial p53 accumulation does not simply result from a passive relocation of p53 to the mitochondria in response to its general up-regulation within the cell. On the contrary, mitochondrial accumulation of p53 can be dissociated from its accumulation within the cell, suggesting that some active mechanism of redistribution is at play. Akt likely plays a critical role in this process. Studies are currently under way in our laboratory to elucidate the precise mechanisms by which Akt influences mitochondrial p53 accumulation.

In summary, the current study establishes a role for Akt in modulating the direct action of p53 on the caspase-dependent mitochondrial death pathway and suggests that these two cell fate determinants interact at the level of the mitochondria to influence CDDP sensitivity (Fig. 8). A thorough understanding of the mechanisms of CDDP resistance may improve treatment outcomes for human ovarian cancer.

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