Trichostatin A up-regulates p73 and induces Bax-dependent apoptosis in cisplatin-resistant ovarian cancer cells

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Abstract

Several studies in the last years evidenced that deregulation of proapoptotic and antiapoptotic pathways are key players in the onset and maintenance of chemoresistance in advanced ovarian cancers. To characterize the signaling events and molecules involved in the acquisition of cisplatin resistance, we used the human ovarian cancer cell line A2780 and its derivative cisplatin-resistant subline A2780 CIS. We found that the mitochondrial intrinsic apoptotic pathway, induced by *cis*-dichlorodiammineplatinum (CDDP) in A2780 wild-type cells, was compromised in the resistant subline CIS. The analysis of expression of proteins involved in mitochondria-dependent apoptosis revealed a role of Bax and p73 but not p53. Indeed, we found that CDDP treatment induced the up-regulation of p53 in both sensitive and resistant A2780 cell lines. By contrast, p73 and Bax expressions were compromised in resistant cells. Pretreatment of resistant A2780 CIS cells with the histone deacetylase inhibitor trichostatin A overcomes apoptosis resistance to CDDP by restoring both p73 and Bax but not p53 expression. Altogether, these data indicate that p73, but not p53, is involved in the regulation of apoptosis susceptibility to cisplatin in A2780 ovarian cancer cells and evidence a key contribution of histone deacetylase activation in the acquisition of chemotherapy resistance in human ovarian cancer cells. [Mol Cancer Ther 2008;7(6):1410–9]

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Introduction

Cisplatin is one of the most effective DNA-damaging agents used for treatment of human ovarian cancer. However, one of the major limits on its efficacy in tumor regression is the frequent acquisition of multidrug resistance associated with decreased susceptibility to apoptosis (1). Similarly to most chemotherapeutic drugs, cisplatin induces apoptosis by activating the intrinsic mitochondrial pathway (2), whose alterations have been found in resistant phenotypes (3–6).

The mitochondrial pathway is regulated by the Bcl-2 family of proteins (7). The Bcl-2 family comprises multidomain proteins with antiapoptotic (Bcl-2, Bcl-xL, Bcl-W, 1, and Bfl-1) and proapoptotic (Bax, Bak, Bid, Bim, and Hrk) functions and a subset of proapoptotic members known as BH3-only proteins with regulatory functions. The BH3-only proteins (Bid, Bad, Bim, Noxa, and Puma) function upstream of the proapoptotic proteins Bax and Bak. Indeed, they communicate both extrinsic (death receptor, growth factor, and T-cell receptor) and intrinsic death stimuli (DNA damage) to Bax and/or Bak favoring their oligomerization, the release of cytochrome *c* from the mitochondrial membrane and subsequently inducing cell death. The ratio of death inducing (Bax) and death-inhibitory members (Bcl-2 and Bcl-xL) determines whether a cell will respond to an apoptotic signal by mediating the disruption of the mitochondrial membrane and the release of caspase activators. Elevated expression of antiapoptotic and/or reduced expression of proapoptotic genes of Bcl-2 family has been reported in cancer (8).

Proapoptotic Bax plays an important role in both inhibiting tumor progression and promoting apoptosis of some tumors in response to DNA-damaging agents. In particular, Bax loss of function has been observed in hematopoietic and colon cancers (9, 10) and reduced *bax* mRNA and protein levels have been also associated with resistance to chemotherapy (11, 12). However, the role of Bax expression and/or regulation in the development of chemotherapy resistance in ovarian cancers remains still undefined.

Transcription of the *bax* gene is controlled by several transcription factors including the tumor suppressor proteins belonging to p53 family (13). *Bax* promoter contains a DNA consensus sequence for binding of p53. Exogenously expressed p53 increases *bax* expression in several cell types and this increase correlates with the induction of apoptosis (14). Recently, another member of the p53 family, p73, with *trans*-activating potential on the *bax* gene promoter has been identified (15). p73 is expressed in multiple isoforms (p73α-ζ) derived from alternative splicing at the 3′ (16). p53 is frequently mutated in cancer and >90% of the mutations occur within its DNA-binding domain, thus leading to the inability of p53 to

trans-activate the target gene promoters, such as p21 and *bax* (17). Although p53 mutations have been identified also in ovarian cancers, their association with acquired resistance to cisplatin-based therapy was not found (18). Unlike p53, inactivating mutations in p73 have not been commonly found in human cancers, including ovarian carcinoma (16). These data suggest that other mechanisms, affecting p53 and/or p73 expressions and functions, may contribute to the development of resistance to cisplatin-induced apoptosis.

By using the human ovarian carcinoma cell line A2780 and its derivative cisplatin-resistant clone A2780 CIS (19), expressing functionally active p53 and p73 proteins, we show that the acquisition of resistance to apoptosis is dependent on defects in cisplatin-induced Bax expression and associated mitochondrial membrane depolarization, cytochrome *c* release, and activation of caspase-3. The analysis of the proteins involved in the regulation of Bax expression revealed a role for p73 but not p53. Indeed, we found that *cis*-dichlorodiammineplatinum (CDDP) treatment induced the up-regulation of p53 in both A2780 wild-type (WT) and CIS cells. By contrast, p73 and Bax expressions were blocked in resistant cells. We also found that the defects of both p73 expression and p73-dependent up-regulation of Bax were associated with the activation of histone deacetylases (HDAC), which are known negative regulators of both p53- and p73-dependent apoptosis (20).

Altogether, these data indicate that p73, but not p53, is a key regulator of apoptosis susceptibility to cisplatin in A2780 ovarian cancer cells and evidence that the activation of HDAC may contribute to cisplatin resistance in human ovarian cancer cells.

Materials and Methods

Cell Lines, Antibodies, and Reagents

The human ovarian carcinoma cell line A2780 WT and its cisplatin-resistant subline A2780 CIS (19) were obtained from European Collection of Cell Cultures and were cultured in DMEM supplemented with 10% FCS, L-glutamine, penicillin, and streptomycin (Life Technologies). To retain cisplatin resistance, 1 μmol/L cisplatin was added to the culture medium of A2780 CIS every two passages. CDDP was obtained from Teva Pharma, trichostatin A (TSA) was purchased from Sigma, and aspirin was from Alexis. Anti-p73 (H-79), anti-p53 (DO-1), anti-Bax (N-20), anti-caspase-3 (H-277), and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies were purchased from Santa Cruz Biotechnology. Anti-cytochrome *c* monoclonal antibody was from BD Biosciences.

Determination of CDDP Cytotoxicity

The cytotoxicity of CDDP on A2780 WT and CIS cells was evaluated by trypan blue assay. Cells were seeded at 100,000/mL in 48-well plates. The following day, cells were treated with different doses of CDDP for further 24 h. Cytotoxicity was determined by quantifying the ability of cells to incorporate trypan blue and expressed as percent mortality. IC_{50} (concentration that inhibits 50% of cell survival) was calculated.

Plasmids, Cell Transfection, and Luciferase Assays

pcDNA3-expressing HA-tagged human p73α and FLAG-p53 have been described previously (21). *Bax*-luciferase reporter construct was obtained by subcloning the PCR-generated fragment (-715 to -317 bp) from the *bax* gene promoter into *Bgl*II-*Hin*dIII sites of the pGL3-luciferase enhancer vector (Promega; ref. 22).

A2780 WT and CIS cells were transiently transfected with the indicated expression vectors by using LipofectAMINE 2000 (Invitrogen) following the manufacturer's instruction. After overnight incubation, luciferase activity was measured according to the manufacturer's instruction (Promega). Transfection efficiency was controlled by coexpressing a plasmid, encoding an enhanced green fluorescent protein (GFP; Clontech). Luciferase activity determined in triplicates was expressed either as fold induction over the basal activity of cells transfected with empty vectors or as arbitrary luciferase units after normalization to GFP values.

Total and Cytoplasmic Extract Preparation and Immunoblotting

Total protein extracts were obtained by lysing cells for 30 min at 4°C in lysis buffer [20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 1% SDS] in the presence of protease and phosphatase inhibitors. For cytochrome *c* release analysis, cytoplasmic extracts were obtained by lysing cells in 200 μL digitonin lysis buffer (75 mmol/L NaCl, 1 mmol/L NaH_2PO_4, 8 mmol/L Na_2HPO_4, 250 mmol/L sucrose, and 190 μg/mL digitonin). After 5 min on ice, cells were centrifuged for 10 min at 14,000 rpm at 4°C and the supernatants, containing the cytosolic fraction, were recovered. Proteins were resolved by SDS-PAGE and blotted onto nitrocellulose membranes. Blots were incubated with the indicated primary antibodies, extensively washed, and, after incubation with horseradish peroxidase–labeled goat anti-rabbit or goat anti-mouse antibodies (Amersham Pharmacia), developed with the enhanced chemiluminescence detection system (Amersham Pharmacia).

Apoptosis Analysis by Propidium Iodide and Annexin V Staining

A2780 WT and CIS cells were cultured for 24 h in the presence or absence of the indicated concentrations of CDDP. Where indicated, cells were pretreated for 2 h with TSA (100 nmol/L) or aspirin (10 mmol/L) before adding CDDP. At the end of incubation, cells were carefully resuspended in PBS containing 0.1% Triton X-100 (Sigma) and 100 units/mL RNase A (Sigma), stained with 50 μg/mL propidium iodide (PI; Sigma), and incubated at 37°C for 15 min. Apoptosis was analyzed by a BD Biosciences FACSCalibur by a biparametric analysis of FL2-H versus SSC-H graphs. Cells showing <2C DNA content (hypodiploid cells) and high SSC-H (granular, highly condensed cells) were regarded as apoptotic. The mean frequencies of apoptotic cells were calculated at least from three independent experiments and statistically analyzed using Student's *t* test. The percentage of specific apoptosis was calculated as follows: % specific apoptosis = 100 × (% induced apoptotic cells - % spontaneous apoptotic

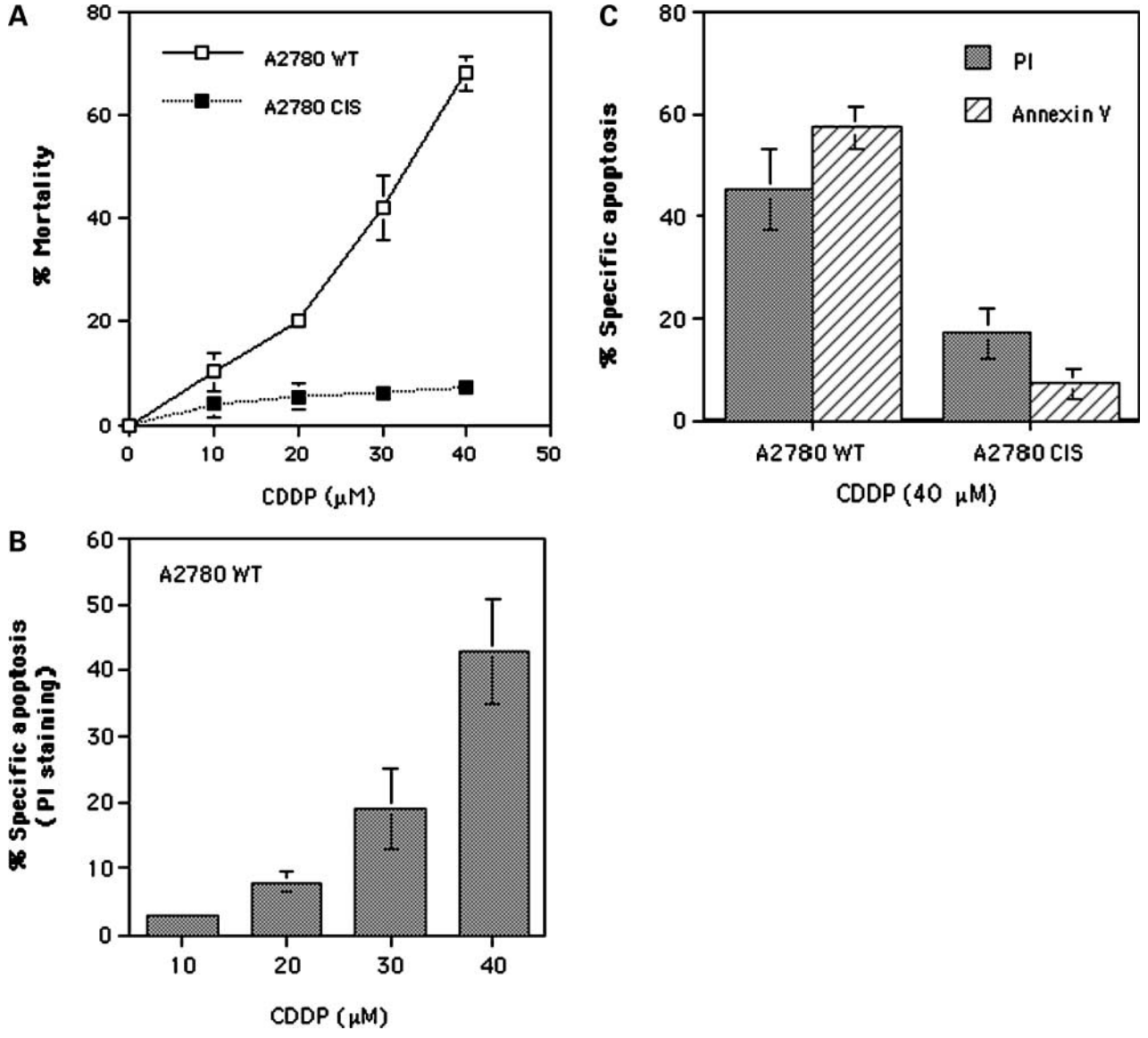

Figure 1. Apoptosis analysis of either sensitive (WT) or cisplatin-resistant (CIS) A2780 ovarian carcinoma cell line. **A,** A2780 WT and CIS cells were treated for 24 h with different doses of CDDP. Cytotoxicity was determined by quantifying the ability of cell to incorporate trypan blue. Mean ± SD percent mortality of three independent experiments. CDDP IC_{50} for A2780 WT cells was 33 μmol/L. **B,** A2780 WT cells were exposed to different doses of CDDP, and after 24 h, apoptosis induction was measured by PI staining and expressed as percentage of specific apoptosis. Mean ± SD of three independent experiments. **C,** A2780 WT and CIS cells were treated for 24 h with 40 μmol/L CDDP. Percent specific apoptosis was evaluated by both PI and Annexin V staining. Mean ± SD of three independent experiments.

cells) / (100 - % spontaneous apoptotic cells). Apoptosis was also evaluated by staining cells with Annexin V conjugates by using a commercially available kit (Molecular Probes).

Analysis of Variation in the Mitochondrial Transmembrane Electrical Potential $\Delta\Psi_m$

Variation in $\Delta\Psi_m$ at the single mitochondrial level was detected by using the lipophilic cation 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1; Molecular Probes). Briefly, cells were incubated at 10^6/mL in RPMI 1640 with 10% FCS for 15 min at 37°C with 10 μg/mL JC-1. At the end of incubation, cells were washed twice and resuspended in PBS, and $\Delta\Psi_m$ was analyzed by a BD Biosciences FACSCalibur flow cytometer. Depolarization of mitochondrial membrane is accompanied by a change of JC-1 color from greenish orange (analyzed in FL-2) to green (analyzed in FL-1).

Small Interfering RNA Transfection

Bax sMART pool small interfering RNA (siRNA) oligonucleotides and scrambled siRNA (control) were purchased from Dharmacon. Cationic lipid complexes, prepared by incubating 100 pmol of the indicated siRNA

with 2 μL LipofectAMINE 2000 in 200 μL Opti-MEM (Life Technologies) for 20 min, were added to adherent A2780 WT cells in a final volume of 2 mL. After overnight incubation, cells were washed and cultured in the presence or absence of 30 μmol/L CDDP for further 24 h. At the end of incubation, Bax expression was analyzed by Western blotting and apoptosis was evaluated by PI staining.

p53 Sequencing Analysis

A2780 WT and A2780 CIS cell lines were sequenced to evaluate the presence of mutations in the p53 coding region. Total RNA was extracted from cells by using the TRI-Reagent solution according to the manufacturer's directions (Molecular Research Center), and after DNase treatment, 1 μg RNA was reverse transcribed with 25 units Moloney murine leukemia virus reverse transcriptase. PCR was done using 2 μL of each cDNA and the AmpliTaq Gold polymerase (Applied Biosystems), and the efficiency was checked in a 1% agarose gel electrophoresis. The following primers were used both for amplification and sequencing: p53-1F 5′-GACACGCTTCCCTGGATTGGC-3′, p53-1R 5′-GCAAAACATCTTGTTGAGGGCA-3′, p53-2F 5′-GTTTCCGTCTGGGCTTCTTGCA-3′, p53-2R 5′-GGTACAGTCAGAGCCAACCTC-3′, p53-3F 5′-TGGCCCCTCCTCAGCATCTTA-3′, p53-3R 5′-CAAGGCCTCATTCAGCTCTC-3′, p53-4F 5′-CGGCGCACAGAGGAAGAGAATC-3′, and p53-4R 5′-CGCACACCTATTGCAAGCAAGGG-3′.

cDNA sequence analysis was done using an automated DNA sequencer (ABI Prism 3100; Applied Biosystems) and the BigDye Terminator v3.1 staining kit (Applied Biosystems) according to the manufacturer's instructions. The results were analyzed using the SeqScape v2.0 software package (Applied Biosystems) using as a reference sequence the Genbank accession no. NM_000546.

Results

Dysfunction in Bax Expression and Mitochondrial Damage Correlate with the Acquisition of Resistance to Cisplatin-Induced Apoptosis in A2780 Ovarian Cancer Cells

Acquisition of resistance to cisplatin-based chemotherapy in ovarian cancer cells has been associated to both caspase-3-dependent (4, 6) and caspase-3-independent (3) pathways, which correlated with either the ability or the inability of the cisplatin-resistant cancer cell lines to activate the apoptotic program, respectively. To circumvent this problem and identify molecular alteration of the apoptotic pathways in resistant ovarian cancer cells, we used the human ovarian carcinoma line A2780 (WT) or its derivative cisplatin-resistant subline (CIS), developed by chronic exposure of the parental A2780 cell line to increasing doses of cisplatin (19). The results obtained by analyzing the cytotoxic activity of different CDDP doses (Fig. 1A) revealed a strong resistance of A2780 CIS when compared with the parental A2780 WT cells (IC_{50}, 33 μmol/L). CDDP strongly induced apoptosis in A2780 WT cells after 24 h (Fig. 1B). By contrast, high doses of CDDP (40 μmol/L) failed to activate the apoptotic program in A2780 CIS cells (Fig. 1C). The mitochondrial membrane depolarization and associated mitochondria damage was evaluated by using JC-1 (Fig. 2A). Depolarization of mitochondrial membrane is accompanied by a change of JC-1 color from greenish orange (FL-2) to green (FL-1). The regions R1 and R2 (Fig. 2A, *top left*) refer to the % of cells with normal (R1) or depolarized (R2) mitochondrial membrane potential. Consistent with the data from apoptosis analysis, the acquisition of resistance to CDDP was also associated to a reduced percentage of cells with depolarized mitochondria membrane potential (Fig. 2A). Depolarization of mitochondrial membrane leads to the release of cytochrome *c* to the cytosol and the subsequent activation of the effector caspase-3 (8). Consistent with the data obtained from the analysis of the mitochondrial membrane potential changes, CDDP treatment induced

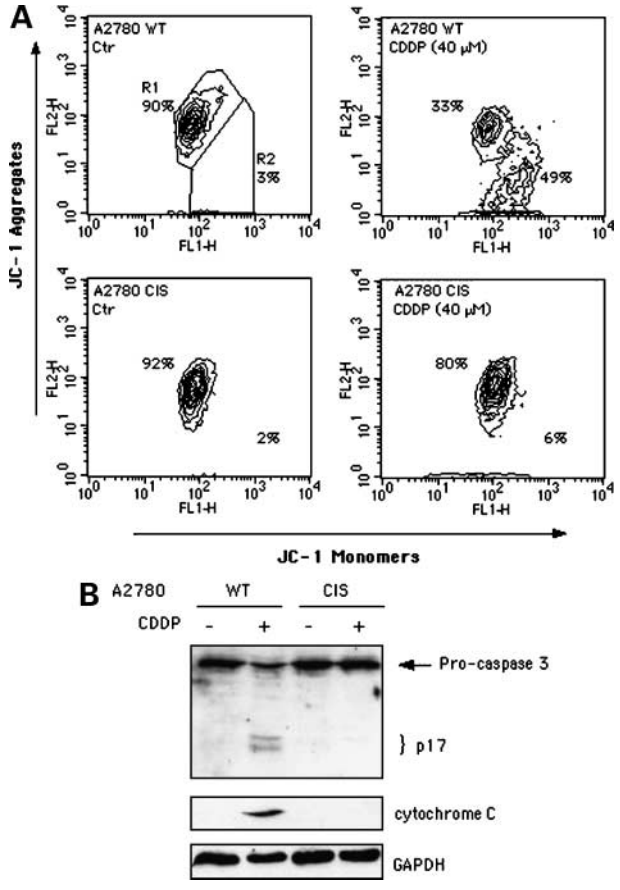

Figure 2. CDDP induces mitochondrial membrane potential depolarization, cytochrome *c* release, and caspase-3 activation in A2780 WT but not in resistant A2780 CIS cells. **A,** A2780 WT and CIS cells were cultured for 24 h in presence or absence of 40 μmol/L CDDP and mitochondrial membrane depolarization was analyzed by flow cytometry after JC-1 staining. **B,** cleavage of procaspase-3 (32 and 17 kDa subunits) and cytoplasmic cytochrome *c* release were detected by Western blotting in both A2780 WT and CIS cells treated for 24 h with 40 μmol/L CDDP. Anti-GAPDH immunoblotting was used as loading control. Representative of one of three independent experiments.

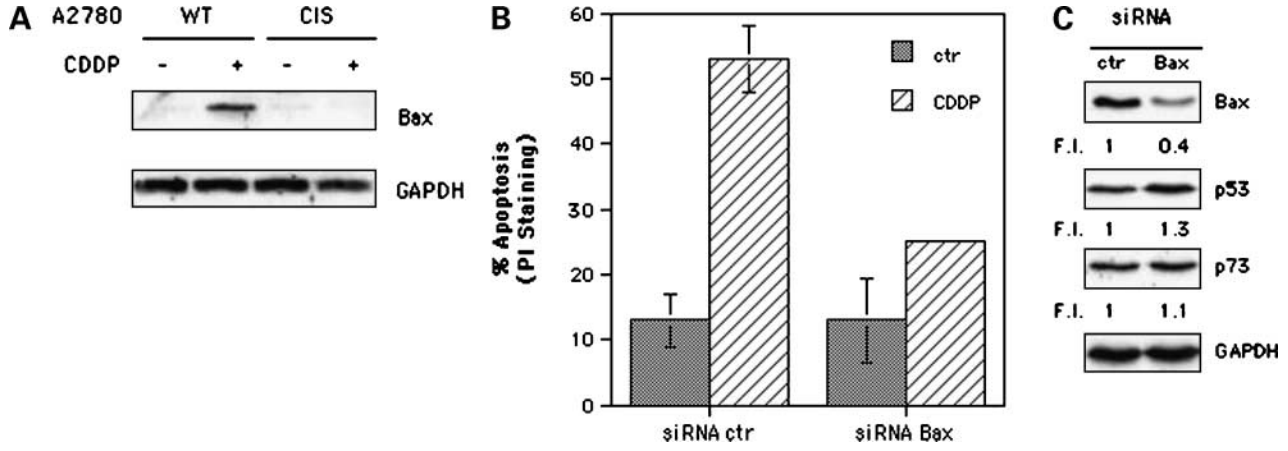

Figure 3. CDDP-induced apoptosis in A2780 depends on efficient Bax expression. **A,** Bax protein expression analysis of either A2780 WT or CIS cells exposed to 40 μmol/L CDDP for 24 h. **B,** A2780 WT cells were transfected with 100 pmol scrambled siRNA (control) or 100 pmol Bax-specific siRNA duplexes by using LipofectAMINE 2000 for 24 h and then cultured in the presence or absence of 30 μmol/L CDDP for further 24 h. Percent apoptosis was evaluated by PI staining. Mean ± SD of three independent experiments. **C,** A2780 WT cells transfected with either control or Bax siRNA and treated with CDDP were analyzed by Western blotting for Bax, p53, p73, and GAPDH expressions. Data were quantified and expressed as fold of induction (*F.I.*) over the basal level after normalization to GAPDH expression (**A – C**). Representative of three independent experiments.

the release of cytochrome *c* into the cytoplasm of A2780 WT cells but not in resistant A2780 CIS cell line (Fig. 2B, *middle*). Cytochrome *c* release was also followed by the activation of effector caspases. Indeed, CDDP induced the cleavage procaspase-3 protein (32 kDa) to its active subunit p17 in sensitive but not in resistant A2780 cells (Fig. 2B, *top*).

The release of cytochrome *c* from the mitochondrial membrane is regulated by members of the Bcl-2 family (7). Bax is directly involved in the release of cytochrome *c* from mitochondria and its induction has also been described to initiate chemotherapy-induced apoptosis in several cancer cell lines (9–12). Therefore, we analyzed both Bax function and expression in A2780 cell lines in response to CDDP treatment. As shown in Fig. 3A (*top*), CDDP treatment induced a strong increase in Bax protein levels in the apoptosis-sensitive A2780 WT but not in the resistant CIS cell line. Protein silencing by oligonucleotide siRNA duplexes specific for Bax significantly reduced (~57% decrease) CDDP-induced apoptosis in the sensitive A2780 WT cells. By contrast, scrambled siRNA (control) did not exert any significant effect (Fig. 3B). The specificity of Bax siRNA duplexes was evaluated by analyzing Bax protein expression in CDDP-treated A2780 WT cells (Fig. 3C). Altogether, these data show evidence that the acquisition of resistance to cisplatin-induced apoptosis in A2780 cells was dependent on defects in both CDDP-induced Bax up-regulation and mitochondrial intrinsic pathway activation.

Acquisition of Apoptosis Resistance in A2780 Cells Is Associated with the Loss of CDDP-Induced Expression of p73 but not p53

Bax expression is controlled by several transcription factors including the tumor suppressor proteins p53 (14) and p73 (13), and both p53 and p73 have been described to exert crucial role in anticancer drug-induced apoptosis (23). Firstly, we analyzed the expression of Bax, p53, and p73 in both A2780 WT and CIS. CDDP treatment induced a significant increase of endogenous p53, p73, and Bax in apoptosis-sensitive WT cells. The loss of Bax expression in resistant CIS cells was accompanied by the absence of CDDP-induced p73 expression but not p53, wherein CIS cells were always induced at higher levels than WT cells (Fig. 4A). To evaluate the ability of expressed p53 and p73 to *trans*-activate the *bax* promoter, we did luciferase assays by using a *bax* luciferase reporter construct containing the luciferase gene under the control of the *bax* promoter (−715 to −317 bp) in the A2780 WT cells. Both p53 and p73α efficiently *trans*-activate the *bax* promoter at a similar extent (Fig. 4B). p53 and p73 overexpression was analyzed by Western blotting (Fig. 4C).

Mutations in p53 have been reported in at least 50% of human cancers, whereas tumor-associated mutations in p73 have not been found (17). Sequencing analysis of p53 in both A2780 WT and A2780 CIS cell lines revealed a single nucleotide polymorphism R72P in both cell lines. One missense mutation, substitution AAG/AAT in heterozygosis yielded K351[K,N], was detected in A2780 CIS cells (Fig. 4D), but as evidenced by the Western blotting data (Fig. 4A), this mutation does not affect p53 expression.

These data strongly support a role for p73 in transducing cisplatin-induced Bax expression and apoptosis in A2780 cancer cells.

TSA Sensitizes Resistant Cells to Cisplatin-Induced Apoptosis by Up-regulating Both p73 and Bax Expressions

p53- and p73-dependent apoptosis is regulated by the opposing activities of histone acetyltransferases (p300CBP) and HDAC (HDAC1/2 and SIRT1; ref. 20). Aberrant activation of HDAC has been associated with both tumorigenesis and chemotherapy resistance also in human ovarian cancer cells (24–26). Therefore, we investigated if

the defects in both CDDP-induced Bax expression and apoptosis in resistant A2780 CIS cells were due to the activation of HDAC. As shown in Fig. 5A, pretreatment with the HDAC inhibitor TSA (27) completely overcomes cisplatin resistance in A2780 CIS cells by restoring CDDP-mediated apoptosis (17% CDDP versus 80% CDDP + TSA). TSA + CDDP IC_{50} for A2780 CIS cells was ~50 μmol/L after 24 h. CDDP-mediated apoptosis in TSA-treated A2780 CIS cells was mitochondria dependent as confirmed by the induction of mitochondrial membrane depolarization (Fig. 5B), caspase 3 activation, and cytochrome *c* release into the cytosol (Fig. 5C). By contrast, treatment with aspirin, an anti-inflammatory drug known to inhibit ovarian tumor cell growth and, in some cases, to activate apoptosis (28, 29), did not exert any significant effects (Fig. 5).

Accumulating evidence suggest that acetylation of both p53 and p73 promotes protein stability, thus preventing their ubiquitin-dependent degradation (30, 31). Therefore, we examined the effects of TSA on the expression of p53, p73, and Bax in CDDP-treated resistant A2780 CIS cells. Consistent with the restoration of mitochondria-dependent apoptosis (Fig. 5), Bax expression strongly increased (~30-fold) following combined treatment of A2780 CIS cells with TSA and CDDP (Fig. 6A, *bottom middle, lane 3* versus *lane 2*). Bax increase was also accompanied by a significant up-regulation of p73 levels (Fig. 6A, *top, lane 3* versus *lane 2*). By contrast, the expression of p53 was down-regulated (~50% reduction) by TSA in CDDP-treated A2780 CIS cells (Fig. 6A, *top middle, lane 3* versus *lane 2*).

In addition to the regulation of the protein half-life, acetylation also affects the *trans*-activating potential of

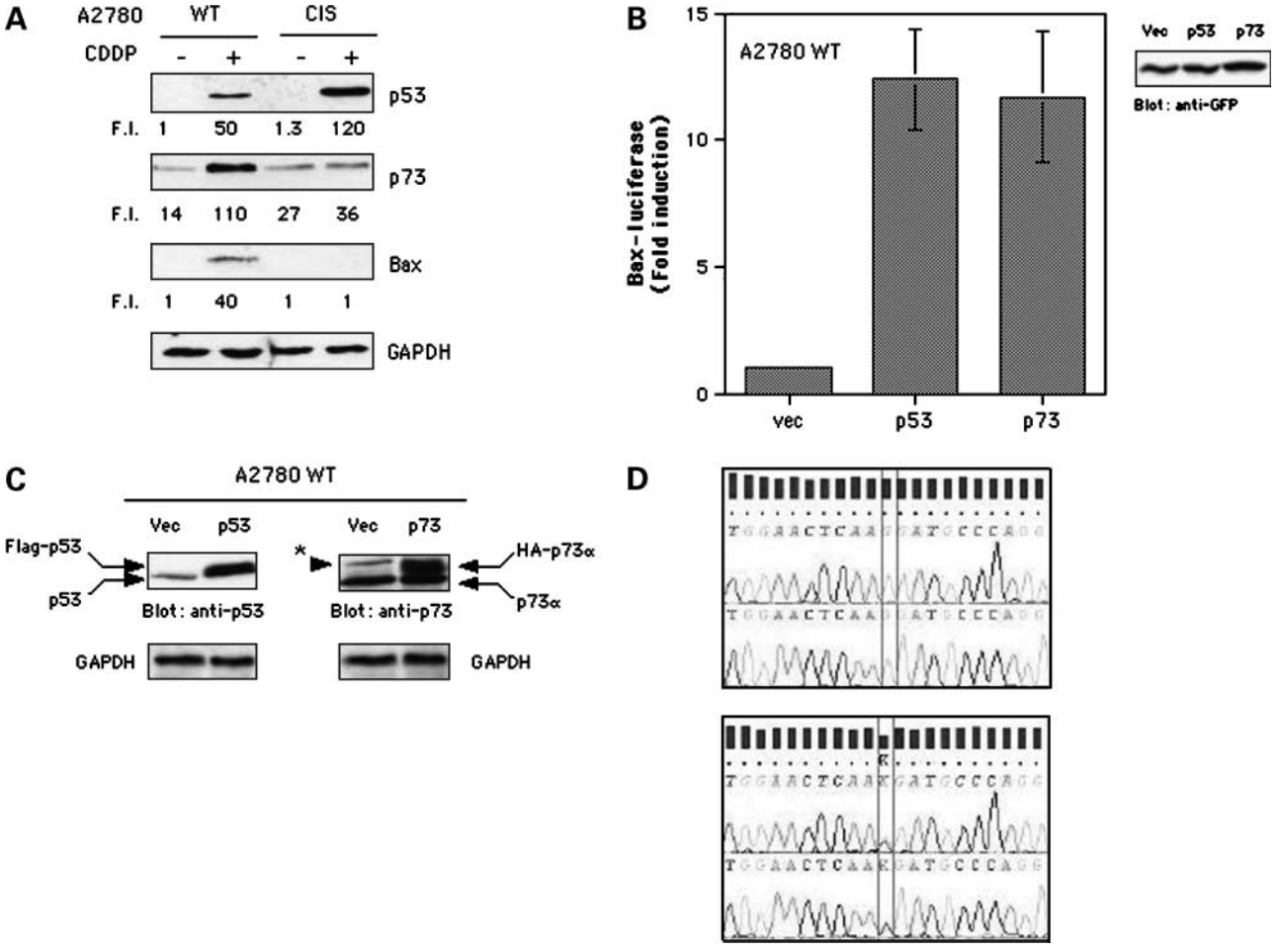

Figure 4. Analysis of p53 and p73 implication in both Bax expression and apoptosis. **A,** A2780 WT and CIS cells were cultured with CDDP 40 μmol/L. After 24 h, Bax, p53, and p73 protein expressions were analyzed by immunoblotting. Data were quantified and expressed as fold of induction over the basal level after normalization to GAPDH expression. Representative of at least three independent experiments. **B,** luciferase activity of A2780 WT cells transfected for 24 h with 1 μg enhanced GFP and 0.4 μg *bax*-luciferase reporter construct together with empty vector (*vec*) or 1 μg Flag-tagged p53 or 1 μg HA-tagged p73α expression vector. Results are expressed as fold induction over the basal activity after normalization to GFP values. Mean ± SD of three independent experiments. GFP expression was analyzed by Western blotting (*right*). **C,** anti-p53, anti-p73, or anti-GAPDH immunoblotting of samples in **B**. The positions of exogenous Flag-p53 or HA-p73α as well as endogenous p53 or p73α are indicated. *Asterisk,* nonspecific band recognized by the anti-p73 antibody. Representative of at least four independent experiments. **D,** *top,* electropherograms of WT sequence in A2780 cell line; *bottom,* electropherograms of substitution K351[K,N] (AAG/AAT) in A2780 CIS cell line. Reference NM_000546.

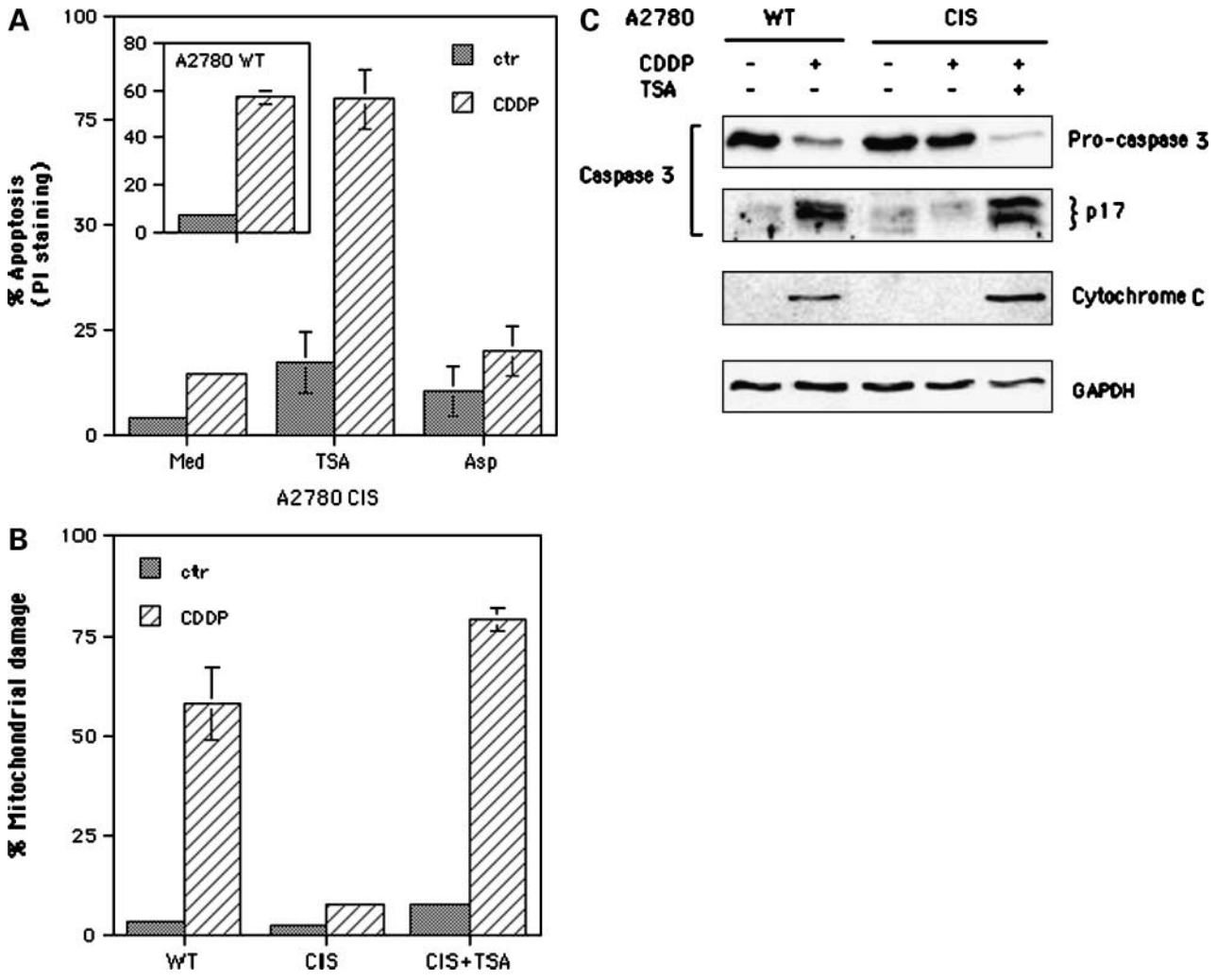

Figure 5. HDAC inhibitor, TSA, sensitizes A2780 CIS cells to cisplatin-induced apoptosis. A2780 CIS cells were pretreated with 100 nmol/L TSA or 10 mmol/L aspirin for 2 h and maintained for further 24 h in the absence or presence of CDDP. **A,** apoptosis was measured by PI staining and expressed as percent apoptosis. *Inset,* percent apoptosis of A2780 WT cells treated for 24 h with CDDP. Mean ± SD of three different experiments. **B,** percent mitochondrial damage was evaluated by measuring mitochondrial membrane depolarization by JC-1 staining. Mean ± SD of three different experiments. **C,** cleavage of procaspase-3 and cytochrome *c* release was detected by Western blotting. Anti-GAPDH immunoblotting was used as loading control. Representative of at least three independent experiments.

both p53 and p73 (32–34). Thus, it is conceivable that if cisplatin resistance is associated to the activation of deacetylases, the *trans*-activity of p73 on the *bax* gene promoter could be compromised in A2780 CIS cells. Indeed, exogenously transfected p73 showed a reduced ability (60% decrease) to *trans*-activate *bax* luciferase in A2780 CIS cells when compared with WT. TSA treatment significantly increased p73-dependent (~90% increase) *trans*-activation of the *bax* promoter (Fig. 6B) without significant changes in p73 expression (data not shown).

Altogether, these data evidence a key role of acetylases in the regulation of CDDP-induced p73 expression and *trans*-activation of the *bax* gene as well as in apoptosis induction in ovarian cancer cells.

Discussion

Apoptosis is frequently deregulated in cancer, as a consequence of increased expression of antiapoptotic proteins or decreased activity of proapoptotic proteins of the Bcl-2 family. In human ovarian cancers, resistance to drug therapy has been often associated to the up-regulation of antiapoptotic Bcl-2 and/or Bcl-xL proteins (6, 35–37). Less is known about the role of Bax in the development of chemotherapy resistance in ovarian cancers, although a correlation between cisplatin resistance and reduced Bax expression has been observed previously in some ovarian carcinoma cells (5, 38), and Bax overexpression has been shown to sensitize ovarian cancer cell lines to drug-mediated apoptosis (39). Here, we show that Bax expression is necessary for CDDP-induced mitochondrial damage

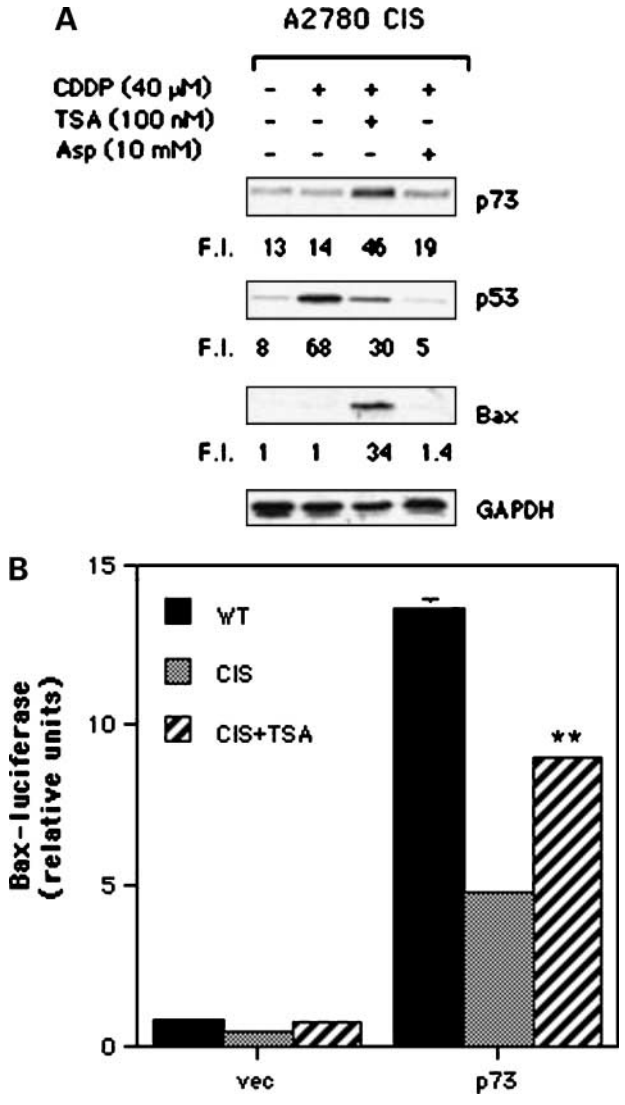

Figure 6. Sensitisation of A2780 CIS cells to CDDP-induced apoptosis by TSA is associated to the recovery of both p73 expression and function. **A,** Western blotting analysis of p73, p53, and Bax expressions of A2780 CIS cells treated for 24 h with the indicated compounds. Data were quantified and expressed as fold of induction over the basal level after normalization to GAPDH expression. Representative of at least three independent experiments. **B,** luciferase activity of A2780 WT and CIS cells transfected for 24 h with enhanced GFP, *bax*-luciferase reporter construct together with empty vector or HA-tagged p73α. Mean ± SD of arbitrary luciferase units after normalization to GFP values. **, $P \leq 0.001$. Representative of at least three independent experiments.

and apoptosis in A2780 cells. Indeed, silencing of *bax* by using specific siRNA oligonucleotides is sufficient to convert cisplatin-sensitive A2780 WT cells in the resistant ones. We also show that the loss of Bax expression in apoptosis-resistant A2780 CIS cells following CDDP treatment is due to defects in the pathways regulating p73-mediated Bax expression.

The *Bax* gene promoter contains a DNA consensus sequence (−486 to −448 bp) for binding of the tumor suppressor protein p53. Exogenously expressed p53 increases *bax* expression in several cell types and this increase correlates with the induction of apoptosis (14). The use of quantitative chromatin immunoprecipitation assays has recently revealed that the *bax* gene promoter binds p53 very weakly when compared with other p53 target genes, such as *Noxa* and *Puma* (40), which have been described as critical mediators of the apoptotic responses induced by p53 (41). The ability of p73 to *trans*-activate several endogenous p53 target genes, including the *bax* gene, has been reported in several cell systems (42, 43). Similarly to p53, p73 also functions in transducing DNA damage signaling to the apoptotic pathway (32, 44). Mutations in p53, affecting its expression as well as its ability to *trans*-activate downstream target genes, such as $p21^{WAF}$ and *bax*, have been identified in at least 50% of human cancers (17). We found a single mutation of p53 (K351N) in resistant A2780 CIS cells that does not affect neither p53 expression (Fig. 4) nor described p53 function (17). These data, together with the observation that CDDP treatment induced a strong up-regulation of p53 protein levels, but not of Bax, in the resistant A2780 CIS cell line, make unlikely a direct role of p53 in both cisplatin-induced Bax expression and mitochondria-dependent apoptosis.

In contrast to p53, loss of expression and/or function mutations of p73 are rare in primary tumors. However, several cytotoxic drugs used clinically, including cisplatin, induce the up-regulation of p73α (45, 46). p73 can induce apoptosis in the absence of p53 and silencing of p73 by specific siRNA strongly reduce cisplatin-induced apoptosis in colon cancers (46). Moreover, loss of p73 induction by CDDP has been observed in a cisplatin-resistant subline of T24 transitional carcinoma (47). Consistently, we found that CDDP induces the expression of p73 only in the sensitive A2780 WT cell line and that the loss of p73 expression observed in the resistant subline CIS is associated with the absence of Bax expression. Up-regulation of ΔNp73 isoforms, which act as potent transdominant inhibitors of both p53 and TAp73, has been also described in ovarian cancer (48). However, we did not find expression of ΔNp73 in A2780 WT as well as CIS cells (data not shown). These data strongly support the importance of p73 in cisplatin-induced activation of the mitochondrial apoptotic pathway and also evidence that failure of induction of p73 expression and function may cause the acquisition of cisplatin resistance.

Histone acetyltransferases, such as p300/CBP, play an important role in regulating p73 accumulation in response to chemotherapeutic agents by both increasing its transcription and protein stabilization. Acetylation of the transcription factor E2F1 in response to genotoxic stress promotes its recruitment on the p73 promoter and its transcriptional activation, ultimately leading to apoptosis of damaged cells (49). Similarly to p53 (50), p73 is itself acetylated by p300/CBP histone acetyltransferase (32), an event that stabilizes and protects p73 from ubiquitin-proteasome-dependent degradation (30). In contrast to p53 (31, 51), deacetylation of p73 by HDAC1/2 has not been described. However, our data on the inhibition of p73

expression in resistant A2780 CIS cells exposed to CDDP (Fig. 4A) and its reversion following pretreatment with the HDAC1/2 inhibitor, TSA (Fig. 6A), strongly suggest that deacetylation of p73 by class I and II HDAC may likely occur in cisplatin-resistant cells, thus leading to the repression of p73 expression and apoptosis. Moreover, our observations that p53 levels are reduced by TSA in CDDP-treated A2780 CIS cells (Fig. 6A) confirm previous data from Strait et al. (24) and reinforce the role of p73, but not p53, in regulating apoptosis susceptibility in response to cytotoxic drugs.

Acetylases and deacetylases function also as coactivators and corepressors of gene transcription (52). The recruitment of p300/CBP together with p73 on the *bax* promoter is necessary for p73-dependent Bax expression (32). By contrast, binding of the corepressor HDAC-1 is associated with the lost of p73 recruitment on the promoter and inhibition of *bax* gene *trans*-activation (22). We found that p73 transcriptional activity on the *bax* promoter is inhibited in cisplatin-resistant cells, and TSA treatment restores p73-dependent transcription of *bax* (Fig. 6B). HDAC inhibitors have been used previously to induce apoptosis in ovarian cancers (24–26). However, the mechanisms and molecular targets of their actions have not been defined. The data presented herein identify p73 as an important target of HDAC inhibitors in inducing mitochondria-dependent apoptosis in cisplatin-resistant cells and provide useful information for the development of combinatorial therapy for the treatment of ovarian cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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