Proapoptotic Protein Smac Mediates Apoptosis in Cisplatin-resistant Ovarian Cancer Cells When Treated with the Anti-tumor Agent AT101

Wenbin Hu, Fang Wang, Jingsheng Tang, Xinyu Liu, Zhu Yuan, Chunlai Nie, and Yuquan Wei

From the State Key Laboratory of Biotherapy and Cancer Center, West China Hospital, Sichuan University, 4 Keyuan Road, Hitech District, Chengdu 610041, China and ChongQing NewFine Biology Technology Co., Ltd., 8 Lingfang Road, Bangqiao District, Chongqing 402460, China

Background: BH3-mimetic molecules possessing potential anticancer activity are able to inhibit antiapoptotic Bcl-2 family proteins. BH3-mimetic molecules possessing potential anticancer activity are able to inhibit antiapoptotic Bcl-2 family proteins. AT101 (R(-)-gossypol), a natural BH3-mimetic molecule, has shown anti-tumor activity as a single agent and in combination with standard anticancer therapies in a variety of tumor models. Here, we report the effect of AT101 on apoptosis in cisplatin-resistant ovarian cancer cells and identify the major molecular events that determine sensitivity. AT101 induced cell apoptosis by activating Bax through a conformational change, translocation, and oligomerization. The inhibition of Bax expression only partially prevented caspase-3 cleavage. However, the gene silencing of Bax had no effect on mitochondrial Smac release. Further experiments demonstrated that Smac reduction inhibited caspase-3 activation and attenuated cell apoptosis. More importantly, the inhibition of Smac or overexpression of XIAP attenuated Bax activation in ovarian cells. Furthermore, our data indicate that the Akt-p53 pathway is involved in the regulation of Smac release. Taken together, our data demonstrate the role of Smac and the molecular mechanisms of AT101-induced apoptosis of chemoresistant ovarian cancer cells. Our findings suggest that AT101 not only triggers Bax activation but also induces mitochondrial Smac release. Activated Smac can enhance Bax-mediated cellular apoptosis. Therefore, Smac mediates Bax activation to determine the threshold for overcoming cisplatin resistance in ovarian cancer cells.

Significance: We provide a theoretical basis for AT101 as a potential therapeutic agent in the treatment of ovarian cancer.

Chemoresistance of ovarian cancer has been previously attributed to the expression and activation of Bcl-2 family proteins. BH3-mimetic molecules possessing potential anticancer activity are able to inhibit antiapoptotic Bcl-2 family proteins. AT101 (R(-)-gossypol), a natural BH3-mimetic molecule, has shown anti-tumor activity as a single agent and in combination with standard anticancer therapies in a variety of tumor models. Here, we report the effect of AT101 on apoptosis in cisplatin-resistant ovarian cancer cells and identify the major molecular events that determine sensitivity. AT101 induced cell apoptosis by activating Bax through a conformational change, translocation, and oligomerization. The inhibition of Bax expression only partially prevented caspase-3 cleavage. However, the gene silencing of Bax had no effect on mitochondrial Smac release. Further experiments demonstrated that Smac reduction inhibited caspase-3 activation and attenuated cell apoptosis. More importantly, the inhibition of Smac or overexpression of XIAP attenuated Bax activation in ovarian cells. Furthermore, our data indicate that the Akt-p53 pathway is involved in the regulation of Smac release. Taken together, our data demonstrate the role of Smac and the molecular mechanisms of AT101-induced apoptosis of chemoresistant ovarian cancer cells. Our findings suggest that AT101 not only triggers Bax activation but also induces mitochondrial Smac release. Activated Smac can enhance Bax-mediated cellular apoptosis. Therefore, Smac mediates Bax activation to determine the threshold for overcoming cisplatin resistance in ovarian cancer cells.

Ovarian carcinoma is the leading cause of death among patients with gynecologic cancer (1). The three most commonly used treatments for ovarian cancer are surgery, radiation, and chemotherapy, with chemotherapy being the predominant mode of treatment. However, as in the treatment of many other cancers, both intrinsic and acquired drug resistance are serious problems in ovarian cancer treatment.

Recent evidence suggests that the failure of drug-induced apoptosis may be an underlying cause of drug resistance. Some studies have identified a number of key mediators of apoptosis that are altered in chemoresistant ovarian cancer cells (2, 3). The Bcl-2 family proteins constitute a central checkpoint for apoptotic pathways. The levels of expression and the activation of the Bcl-2 family proteins often play important roles in controlling apoptotic responses to drug treatments, thus modulating the chemosensitivity of tumor cells (4, 5). These proteins can form multiple BH-3-mediated heterodimeric complexes that modulate their pro- or antiapoptotic properties (6).

These interactions occur via the BH3 domain, which plays a central role in the control of apoptosis. The ratio between pro- and antiapoptotic members, their conformation, and their subcellular localization constitute major determinants of cell survival. Following an apoptotic stress event, BH3-only proteins translocate to the mitochondria and initiate the mitochondrial cell death pathway by either directly activating Bax-like proteins or indirectly binding prosurvival Bcl-2 family members, thereby releasing the Bax-like proteins (6, 7).

Therefore, in ovarian carcinoma, inhibition of the protective function of Bcl-2 and Bcl-xL to release Bax-like proapoptotic multidomain members seems to be an attractive strategy for either restoring the normal apoptotic process or circumventing resistance to conventional chemotherapy. In this regard, many groups have designed BH3 mimetics that bind and inhibit the function of the antiapoptotic members (8).

AT101, a natural product from cottonseed with a BH3-mimetic structure, was identified as a small molecule inhibitor of Bcl-2/Bcl-xL/Mcl-1 that potently induces apoptosis in various cancer cell lines (9). AT101 is one of the world’s first small molecule Bcl-2 inhibitors that has entered into clinical trials and is now in phase II clinical trials for hormone-refractory prostate cancer and other types of cancers (10, 11). Preclinical

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‡ Both authors contributed equally to this work.

‡1 To whom correspondence should be addressed. Tel.: 86-28-85164063; E-mail: niecl1022@hotmail.com.
studies revealed that gossypol not only interrupts the interaction between anti- and proapoptotic Bcl-2 family proteins but also induces BH3 protein (such as Puma and Noxa) up-regulation (9) or down-regulates XIAP expression (12). Thus, gossypol can induce apoptosis by activating apoptogenic factors other than the Bcl-2 family.

The small mitochondria-derived activator of caspases (Smac), also known as direct inhibitor of apoptosis protein (IAP)3 binding protein with low isoelectric point (DIABLO), is a mitochondrial protein containing an N-terminal 55-amino acid mitochondrial import sequence, which is released from mitochondria into the cytosol in response to apoptotic stimuli. Smac promotes caspase activation, presumably by antagonizing IAPs, such as X-linked IAP (XIAP) (13). Recent studies have shown that mitochondrial Smac release is suppressed by Akt, Bcl-2, and Bcl-xL but promoted by Bax, Bad, and Bid (13). Expression of Smac sensitizes human cancer cells to apoptosis induced by a variety of anticancer agents (14), and Smac release is a determinant of chemosensitivity in ovarian cancer cells (15). Recent research demonstrates that gossypol-activated proteins are involved in Smac release (16). However, the effect of Smac on gossypol-induced apoptosis in cisplatin-resistant ovarian cancer cells has not been thoroughly elucidated.

In the present study, we show that Bax was activated during AT101-induced apoptosis of cisplatin-resistant cells. The down-regulation of Bax did not completely inhibit caspase-3 cleavage and did not cause an increase in caspase-3-XIAP interaction. Moreover, knockdown of Bax did not inhibit mitochondrial Smac release or the Smac-XIAP interaction induced by AT101, whereas Smac reduction attenuated Bax activation in ovarian cancer cells. Further experiments indicated that knockdown of Smac inhibited caspase-3 cleavage and partially prevented cytochrome c (cyt c) release following AT101 treatment. Bax and Smac double knockdown inhibited cyt c release. Furthermore, AT101-induced Smac release was dependent on p53 mitochondrial translocation, which was regulated by Akt. These results suggest that Smac potentiates Bax activation and that Smac-mediated Bax activation is a major molecular event in AT101-induced apoptosis in chemoresistant ovarian cancer cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—AT101 was obtained from Ascenta Therapeutics, Inc. (San Diego, CA). PI and Bax (clone 6A7, B8429), FLAG (clone M1, F3040), and actin (clone AC-74, A5316) antibodies were obtained from Sigma. Puma (catalog no. 4976), Bim (catalog no. 2819), XIAP (catalog no. 2042), caspase-9 (catalog no. 9502), Smac (catalog no. 2954), PARP (clone 46D11, catalog no. 9532), p53 (clone 7F5, catalog no. 2527), phospho-Akt (Ser-473) (clone 587F11, catalog no. 4051), Akt (catalog no. 9272), and caspase-3 (clone 8G10, catalog no. 9665) antibodies were purchased from Cell Signaling (Beverly, MA). Mcl-1 (sc-69839), Noxa (sc-30209), Bcl-2 (sc-130307), Bcl-xL (sc-8392), cytochrome c (sc-13156), and Bax N-20 (sc-493) antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The Smac-N7 peptide (AVPAQKPRQIKWFQNRMRK-WKK) and control peptide were purchased from Calbiochem. The Smac-N7 peptide was modified to be cell-permeable by linkage of the COOH-terminal lysine to the arginine of an Antennapedia homeodomain 16-mer peptide via a prolene linker.

**Gene Silencing with Small Interfering RNAs and Plasmids**—Small interfering RNA (siRNA) oligonucleotides were purchased from Dharmacon (Lafayette, CO) with sequences targeting Bax (5'- AACUGAUCAAGAACAAUCUGG-3'), Smac (5'-AACCCUGUGCGGCUUUCAUAU-3'), and p53 (5'-CGGCAUGAACGGGGCCCAU-3'). For Bax shRNA construction, the Bax siRNA was cloned into the pSilencer 2.1-U6 hygro plasmid. Dominant negative Akt1 was a gift from Dr. Michael J. Quon (University of Maryland, Baltimore, MD). The constitutively active Akt1 construct HA-PKB-T308D/S473D was obtained as described previously (17). Smac and XIAP constructs were generated by RT-PCR from total RNA isolated from A2780/CP cells (cisplatin-resistant version of the A2780 cells) and cloning of the RT-PCR products into the pFLAG-CTC vector (Sigma).

**Cell Culture and Transfection**—Cells were obtained from the American Type Culture Collection. OVCAR-3 cells were cultured in suspension with RPMI 1640 medium (Sigma) supplemented with 10% fetal bovine serum (FBS) (Hyclone) and 1% penicillin-streptomycin at 37 °C under 5% CO2. A2780 cells were incubated in DMEM supplemented with 10% FBS and penicillin-streptomycin. OVCAR-3 cells were obtained from patients who were refractory to platinum-based combination chemotherapy (18). Cisplatin-resistant cells were obtained by iterative treatments with increasing concentrations of cisplatin (19). Briefly, cisplatin-resistant ovarian cells were obtained by exposing the cells to 5 μg/ml cisplatin under normal culture conditions. Despite massive cell death concurrent with treatment, the cultures were maintained for 4–6 weeks by regular changes of culture medium until the surviving cells recovered a normal growth pattern. Next, cisplatin treatment was resumed with elevated drug concentrations (7.5–10 μg/ml). After selection, the cisplatin-resistant variants were treated with cisplatin every month to maintain their high level of chemoresistance.

For transfection, cells were seeded on 6-well plates and then transfected with the appropriate plasmid DNA or siRNA using the manufacturers’ protocols. Typically, cells were seeded on coverslips in the 6-well plates, and then 1 μg of plasmid DNA or 100 nM siRNA and 4 μl of DMRIE-C reagent (Invitrogen) were used per coverslip. The cells were incubated for 4 h in the transfection mixture, which was then replaced with fresh culture medium. For stable transfection, cells were transfected with the constructs as described previously (20). Positive clones were selected with 1 mg/ml G418 for several weeks.

**Cell Viability and Apoptosis Assays**—Ovarian cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in triplicate using a standard procedure (21). Four methods were used to assess AT101-induced apoptotic cell death: detection of DNA fragmentation with the Cell Death Detection ELISA kit (Roche Applied Science), Western blot analysis of caspase activation,
PARP cleavage, and measurement of apoptotic cells by flow cytometry (PI staining for sub-G1). The Cell Death Detection ELISA quantified the apoptotic cells by detecting the histone-associated DNA fragments (mono- and oligonucleosomes) generated by the apoptotic cells (17).

Cell Fractionation—Mitochondria and cytoplasm from cells were fractionated by differential centrifugation as described previously (20, 22). Briefly, cells were harvested and resuspended in 3 volumes of hypotonic buffer (210 mM sucrose, 70 mM mannitol, 10 mM Hepes, pH 7.4, 1 mM EDTA) containing 1 mM phenylmethylsulfonyl fluoride, 50 mg/ml trypsin inhibitor, 10 mg/ml leupeptin, 5 mg/ml aprotinin, and 10 mg/ml pepstatin. After gentle homogenization with a Dounce homogenizer, cell lysates were centrifuged at 1,000 × g for 5 min to remove unbroken cells and nuclei. The supernatant was collected and centrifuged at 10,000 × g to pellet the mitochondria-enriched heavy membrane fraction. The supernatant was further centrifuged at 100,000 × g to obtain a cytosolic fraction.

Immunoprecipitation and Immunoblotting—Immunoprecipitation and immunoblotting were performed as described (20). For immunoprecipitation, all cells were harvested by resuspension in CHAPS cell extract buffer (Cell Signaling) and

FIGURE 1. AT101 induces cell apoptosis in ovarian cancer cells regardless of their chemosensitivity. A, analysis of cell viability treated with AT101. Ovarian cancer cells were treated with AT101 at different concentrations for 48 h, and cell viability was assessed by an MTT assay. Graphs show the results of the MTT assay (n = 3, mean ± S.D. (error bars)). B, analysis of cell apoptosis treated with AT101. Cells were treated with AT101 (10 μM) for different periods of time and then collected to examine apoptosis. Cell apoptosis was quantitatively detected by a cell death ELISA kit as described under “Experimental Procedures.” Graph show the results of quantitative analyses (n = 3, mean ± S.D.). C, cells were treated with AT101 (10 μM) for different periods of time and then lysed for immunoblotting with antibody specific for caspase-9 and PARP. CF, cleaved fragments. β-Actin was used as a protein loading control. D, detection of cell apoptosis with flow cytometry. Cells were treated with AT101 (10 μM) for 24 h and then collected for PI staining. Sub-G1 cells (apoptotic cells) were assessed by flow cytometry. Representative results of three experiments with consistent results are shown.
sonicated on ice. Lysates were centrifuged at 14,000 × g at 4 °C for 15 min. Cytosolic extracts were precleared and incubated with antibodies against XIAP, Smac, or Bax with protein A-Sepharose (Invitrogen) to pull down immune complexes. The Sepharose was washed three times with lysis buffer and two times with PBS. Cytosol, mitochondria extracts, total lysates, and immunoprecipitates were analyzed by Western blot with antibody dilutions as follows: actin at 1:20,000; Puma, Bim, XIAP, p53, phospho-Akt (Ser-473), and Akt at 1:2,000; and caspase-9, caspase-3, PARP, Mcl-1, Noxa, Bcl-2, Bcl-xL, Bax cyt c, and Smac at 1:1,000.

**Immunostaining**—A2780/CP cells were seeded in 24-well plates with Lab-Tek chamber slides with a cover (Nalge Nunc International, Naperville, IL) in 500 μl of medium and incubated overnight. Cells were then treated with AT101 (10 μM) for 24 h, and treated cells were incubated with 200 nM MitoTracker Red CMXRos (Molecular Probes, Inc., Eugene, OR) in culture medium for 30 min. Medium was removed, and cells were fixed in 4% formaldehyde containing 0.1% glutaraldehyde for 15 min at room temperature. After rinsing with cold PBS (pH 7.4), cells were permeabilized with 0.5% Triton X-100 for 10 min at room temperature. After blocking with 5% goat serum, antibodies against p53 (Ab-11, Oncogene) (1:70 dilution), cytochrome c (7H8.2C12, BD Pharmingen) (1:100 dilution), Smac (Cell Signaling) (1:100 dilution), or Bax (6A7, Sigma) (1:100 dilution) were added, and the fixed cells were

FIGURE 2. AT101 induces Bax activation in apoptosis. A, time-dependent analysis of expression levels of Bcl-2 members in cells treated with AT101 (10 μM) at the indicated time. Cells were treated and collected and then lysed in Nonidet P-40 buffer for Western blot detection. β-Actin was used as a protein loading control. B, cells were treated with AT101 as described in A and subjected to subcellular fractionation. The cytosolic or mitochondrial fractions were immunoblotted (30 μg of protein/lane) with antibody specific for Bax N-20 (sc-493) (Santa Cruz Biotechnology, Inc.). C, cells were treated with AT101 as described in A for detection of Bax oligomerization. Top, cells were treated with AT101 for 48 h. Fractions (0.6 ml each) were collected, and a portion (20 μl) of fractions 1–19 was analyzed by Western blotting with anti-Bax N-20 antibody (sc-493) (Santa Cruz Biotechnology, Inc.). Bottom, the oligomerization of Bax was assessed by cross-linking with disuccinimidyl suberate as described under “Experimental Procedures.” The samples were then solubilized in Nonidet P-40 lysis buffer, and Bax was detected by Western blotting with anti-Bax N-20 antibody (sc-493) (Santa Cruz Biotechnology, Inc.). D, detection of the conformational change of Bax. Cells were cultured in the presence or absence of AT101 (10 μM) for 48 h and then lysed in CHAPS buffer and subjected to immunoprecipitation (IP) with anti-Bax 6A7 antibody (Sigma). A cell lysate obtained by Nonidet P-40 was used as a positive control. Total lysates were also applied directly onto SDS-PAGE, transferred to blots, and probed with the indicated antibodies, including a specific anti-Bax antibody (sc-493) (Santa Cruz Biotechnology, Inc.). E, immunostaining of Bax translocation and conformational change in A2780/CP cells. A2780/CP cells were treated with or without AT101 (10 μM) at the indicated time, and then cells were fixed and immunostained for Bax translocation and conformational change as described under “Experimental Procedures.” The mitochondria were fluorescently stained with 50 nM MitoTracker. Nuclei were counterstained with DAPI. The arrows indicate conformational change and translocation of Bax. Representative results of three experiments with consistent results are shown.
incubated with antibodies at 37 °C for 1 h followed by incubation with anti-mouse IgG-FITC (Sigma) (1:128 dilution) for 1 h. After removal of antibodies, cells were rinsed with PBS and mounted with 90% glycerol. Fluorescence was immediately observed using an Olympus DP72 microscope.

**Bax Oligomerization**—Bax oligomerization with cross-linking was detected as described previously (20). Cells were washed with conjugating buffer containing 150 mM NaCl, 20 mM Hepes (pH 7.2), 1.5 mM MgCl<sub>2</sub>, and 10 mM glucose. Disuccinimidyl suberate in DMSO was added from a 10-fold stock solution to a final concentration of 2 mM. The samples were incubated at room temperature for 30 min with non-reducing buffer, and the cross-linker was then quenched by the addition of 1M Tris-HCl (pH 7.5) to a final concentration of 20 mM and incubation at room temperature for 15 min. The samples were then solubilized in 0.5% Nonidet P-40 lysis buffer without a reducing agent and centrifuged at 12,000 × g for 10 min. Bax was detected by Western blot using a Bax polyclonal antibody.

For gel filtration analysis of oligomerization, cell lysates were obtained 18–20 h after transfection by lysing the cells in lysis buffer (Buffer A with 2% CHAPS). The composition of Buffer A was 20 mM HEPES-KOH (pH 7.5), 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM dithiothreitol (DTT), and 0.1 mM PMSF supplemented with protease inhibitors (5 mg/ml pepstatin A and 10 mg/ml leupeptin). The cell lysates were incubated for 1 h at 4 °C with rotation before centrifugation for 1 h at 100,000 × g at 4 °C. The supernatant, ~1 mg of total protein as measured by a protein assay (Bio-Rad), was loaded onto an FPLC Superdex 200 10/30 column equilibrated with 100 mM NaCl in Buffer A (with 1.8% CHAPS) and calibrated with the calibration kit provided by the manufacturer (Amersham Biosciences). The proteins were eluted at 0.4 ml/min, and fractions (0.6 ml) were collected using a fast protein liquid chromatography instrument.

**Statistical Analysis**—Statistical analysis of the differences between the groups was performed using Student’s t test with p < 0.05 considered statistically significant.

**RESULTS**

**AT101 Induces Apoptosis in Ovarian Cancer Cells**—We first determined the growth-inhibitory effect of AT101 on various cisplatin-sensitive (A2780) and -resistant (A2780/CP and OVCAR-3) human ovarian cell lines. As depicted in Fig. 1A, AT101 caused a dose-dependent reduction of cell viability in all ovarian cancer cells regardless of their chemosensitivity. To examine AT101-induced apoptosis, the cells were treated with 10 μM AT101, and apoptosis was confirmed by a DNA fragmentation ELISA at various time points. These results demonstrated that AT101 effectively induced apoptosis in cisplatin-sensitive and -resistant ovarian cells (Fig. 1B). Western blots confirmed cell death through detection of caspase-9 and PARP cleavage in all ovarian cells (Fig. 1C). Flow cytometry analysis
with PI staining further revealed that there was no difference in AT101-induced apoptosis between cisplatin-sensitive and -resistant cells (Fig. 1D).

**Bax Activation Is Necessary but Not Sufficient for AT101-induced Cell Apoptosis**—To determine whether AT101 modulates the expression level of proteins important in the survival of ovarian cancer cells, we used Western blot analysis. We focused on the expression of anti- and proapoptotic Bcl-2 family proteins because these proteins are the main targets of gossypol (10). AT101 did not affect the expression of antiapoptotic Bcl-xL, Bcl-2, or Mcl-1. Similar results were found for Bim and Bax induction after treatment with AT101. Although AT101 upregulated Puma and Noxa expression to some extent, no apparent trend was found for protein expression (Fig. 2A).

A recent report indicated that Bax apoptotic activity results mainly from its mitochondrial translocation, oligomerization, and conformational change (20). To determine whether these changes in Bax also occur following AT101 treatment, ovarian cancer cells were incubated with AT101 followed by separation of the mitochondrial and cytosolic fractions. As Fig. 2B illustrates, after treatment with AT101, the level of Bax in the cytosol decreased, and it translocated to mitochondria in a time-dependent manner. We next examined Bax oligomerization. Cross-linking and gel filtration analysis showed that oligomerized Bax existed after AT101 treatment (Fig. 2C). We then analyzed the conformational change of Bax during apoptosis using a Bax 6A7 monoclonal antibody that specifically recognizes the conformational change (20). Following AT101 treatment, the

![Figure 2](image-url)
Smac Mediates Bax-dependent Apoptosis

Bax conformational change was detected in cells (Fig. 2D). To validate Bax activation in apoptosis, we used A2780/CP cells as a model to detect Bax by immunofluorescence with the Bax 6A7 antibody. AT101 indeed induced the Bax conformational change and translocation (Fig. 2E). These results indicate that Bax activation is involved in AT101-induced apoptosis.

To further investigate the contribution of Bax in cell death, we knocked down Bax expression in A2780 and A2780/CP cells. As shown in Fig. 3A, Bax siRNA effectively decreased Bax expression but did not have a major effect on the induction of PARP (caspase substrate) cleavage after AT101 treatment. Meanwhile, upstream caspase-3 activation was not completely prevented in Bax knockdown (KD) cells following AT101 treatment (Fig. 3B). Similar results were found with Bax KD in A2780/CP cells (data not shown). These results suggest that Bax is probably independent of or at least not absolutely required for AT101-induced cell death.

Because some studies demonstrated that the XIAP-caspase-3 interaction is abrogated to promote apoptosis and that Bax deficiency maintained the binding between XIAP and caspase-3 (23, 24), we then determined whether Bax knockdown affects the XIAP-caspase-3 interaction. Positive control immunoprecipitation experiments proved that XIAP binds to caspase-3 in HCT116 cells, whereas XIAP did not bind activated caspase-3 in Bax wild type (WT) or KD ovarian cancer cells, indicating that Bax deficiency does not affect the caspase-3-XIAP interaction (Fig. 3B).

Previous results demonstrated that Bax activation induced Smac release from mitochondria and that Smac neutralized the inhibitory activity of IAPs, including XIAP, and allowed caspase activation during cell death (23). We therefore examined whether Smac was involved in AT101-induced procaspase-3 cleavage and enzyme activity. The association between Smac and XIAP was analyzed by immunoprecipitation of cytostol extracts obtained from Bax WT and Bax KD cells before and after AT101 treatment. Analysis of whole cell lysates showed that expression of Smac and XIAP was not affected by AT101 treatment (Fig. 3C). These results indicate that the release of Smac from mitochondria to the cytosol enables Smac to interact with XIAP to disrupt the XIAP-caspase-3 interaction, thus allowing caspase-3 cleavage and functional activation. The XIAP-Smac interaction is not dependent on Bax activation in ovarian cell death. Moreover, these results suggest that Smac is probably necessary for AT101-induced cell apoptosis.

Smac Plays Important Role in AT101-induced Apoptosis—

We then examined whether the release of Smac is necessary for apoptosis in ovarian cancer cells using the A2780 and A2780/CP cell lines. We first examined mitochondrial and cytosolic Smac levels after AT101 treatment. As shown in Fig. 4A, exposure of ovarian cancer cells to AT101 decreased mitochondrial Smac and increased cytosolic Smac levels in a time-dependent fashion. Similar results were found for cyt c release (Fig. 4A). The release of Smac or cyt c in apoptosis was also consistently shown by immunostaining (Fig. 4B).

To further study the role of Smac in cell apoptosis, we used siRNA to knockdown Smac expression. As depicted in Fig. 5A, a decrease in Smac expression was confirmed by Western blot. Silencing of Smac significantly inhibited AT101-induced caspase-3 cleavage and attenuated apoptosis in these cells (Fig. 5A). Moreover, overexpression of a Smac heptapeptide (Smac-N7) enhanced AT101-induced cell death (Fig. 5B). These results suggest that Smac, the mitochondrial apoptogenic protein, plays a prominent role in the execution of AT101-induced apoptosis in ovarian cancer cells.

Smac Mediates Bax Activation in Cell Apoptosis—Our data already demonstrated that Smac reduction prevented caspase-3 activation. Recent studies revealed that Smac...
could influence cyt c release in cell apoptosis (24, 25). However, it was unclear whether Smac also mediates cyt c release in AT101-induced apoptosis. To determine the relationship between Smac activation and cyt c release in ovarian cells, A2780 and A2780/CP cells were transfected with Smac siRNA and then treated with AT101 at the indicated time. The release of cyt c decreased in Smac siRNA-treated cells after AT101 treatment (Fig. 6, A and B), accompanied by the inhibition of caspase-3 activation (Fig. 6A). These results indicate that Smac can induce cyt c release in AT101-induced apoptosis.

Our previous data demonstrated that Bax activation had no effect on Smac release (Fig. 3C). Conversely, it is unknown whether Smac affects Bax activation in cell death. To address this possibility, we examined the effect of Smac knockdown on Bax activation. As shown in Fig. 7A, AT101 induced large scale Bax oligomerization in control (Ctrl) siRNA cells. By comparison, AT101-treatment failed to activate Bax efficiently in Smac siRNA cells, and only a trace amount of active Bax dimer and trimer was detected. Moreover, the Bax conformational change and mitochondrial translocation were strongly decreased in Smac siRNA-treated cells after AT101 treatment. These findings suggest that Smac can mediate Bax activation in AT101-treated cells.

We then transfected a full-length Smac (FLAG-tagged at the C terminus) into Bax WT or KD ovarian cancer cells. Smac is expressed as a 239-amino acid precursor molecule. The N-terminal 55 residues contain the mitochondria-targeting sequence and are removed after import into mitochondria (23). Subcellular fractionation analysis revealed that transfected Smac was localized to mitochondria and released from mitochondria into the cytosol after AT101 treatment. When a full-length Smac cDNA was transfected into Bax KD cells, AT101-induced apoptosis was restored, as indicated by an increase in PARP cleavage and cyt c release (Fig. 7B), and apoptosis was enhanced in Bax WT cells with full-length Smac following AT101 treatment (Fig. 7B). These data demonstrate that Smac release was necessary for Bax activation.
Our data indicate that Smac is involved in Bax activation. We would expect that Smac-mediated XIAP inhibition is also involved in Bax-mediated apoptosis. To test this possibility, we examined whether transfected XIAP could affect apoptosis. XIAP overexpression significantly inhibited caspase-3 cleavage, although it had no effect on Smac expression or release in AT101-treated cells. In contrast, caspase activation was not affected in cells with the control vector. Moreover, XIAP overexpression also decreased Bax oligomerization (Fig. 7D), translocation, and conformational change (data not shown) in AT101-treated cells.

Akt Inhibits p53-mediated Mitochondrial Smac Release in Cell Apoptosis—Although we confirmed that Smac potentiates Bax activation in AT101-induced apoptosis, the mechanism by which AT101 induces Smac release is not understood. Recent studies demonstrated that p53 had a direct apoptotic role at the mitochondria (15, 26) and that mitochondrial p53 induced Smac release in ovarian cancer cells (15). To determine the relationship between mitochondrial p53 accumulation and AT101 sensitivity in ovarian cancer cells, A2780 and A2780/CP cells were treated with AT101 at the indicated time. Mitochondrial fractions and whole cell lysates were analyzed. As shown in Fig. 8A, p53 accumulated in mitochondria in response to AT101, but there was little change in the expression level of whole cell p53 in all cells. The increase in p53 in the mitochondria following AT101 treatment was confirmed by immunostaining, shown by colocalization of p53 with the mitochondrial marker Mitofusin.
Tracker (red fluorescence) (Fig. 8B), as described previously (27, 28).

To further confirm the effect of p53 on Smac release, we then knocked down p53 with siRNA (p53 siRNA) in A2780 and A2780/CP cells. The p53 siRNA decreased p53 expression but did not induce Smac release on its own. We observed that AT101 combined with p53 siRNA significantly decreased Smac release in ovarian cancer cells (Fig. 9A). These results demonstrate that p53 can induce mitochondrial Smac release.

Our data show that mitochondrial Smac was triggered by p53. Recent studies demonstrated that Akt blocked mitochondrial p53 accumulation and that Akt-regulated mitochondrial Smac release was dependent on p53 function (15). However, it is unclear whether Akt regulates p53-dependent Smac release in AT101-induced apoptosis. To examine the effect of Akt on p53-mediated Smac release, we first determined the phosphorylation level of Akt in treated cells and found that AT101 induced a time-dependent decrease in phosphorylated Akt (p-Akt) in ovarian cells (Fig. 9B).

We then transfected a constitutively active Akt1 gene into A2780/CP cells and examined the release of Smac and cell apoptosis after AT101 treatment. AT101 induced mitochondrial p53 accumulation with Smac release and caspase-3 activation in A2780/CP-Ctrl cells, but these effects were markedly reduced in A2780/CP-Akt1 cells (Fig. 9B). Nuclear staining and flow cytometry analysis revealed that overexpression of Akt1 decreased cell apoptosis (supplemental Fig. 1). In A2780/CP cells transfected with a dominant negative Akt (A2780/CP-DN-Akt), p53 accumulation, Smac release, and caspase-3 activation all increased relative to control cells. Apoptosis also increased in A2780/CP-DN-Akt cells (supplemental Fig. 2). To further ascertain whether Akt mediates Smac release through p53 activation, we transfected A2780/CP-DN-Akt cells with p53 siRNA. The p53 reduction significantly attenuated Smac release and cell apoptosis in AT101-treated cells (Fig. 9B and supplemental Fig. 3), whereas AT101 induced Smac release in control cells as described. These findings indicate that Akt inhibits p53-dependent mitochondrial Smac release by preventing p53 mitochondrial accumulation.

DISCUSSION

AT101 is a natural small molecule antagonist for both Bcl-2 and Bcl-XL and triggers apoptosis in multiple cancer cell lines with overexpression of Bcl-2 and Bcl-XL (9, 29, 30). Few side effects of gossypol have been reported (31), with the major side effects of gossypol being nausea and vomiting in the third

FIGURE 8. AT101 induces p53 mitochondrial translocation. A, cells were treated with AT101 for different times and then subjected to subcellular fraction for immunoblotting detection of p53 translocation. β-Actin and Cox IV were used as a protein loading control. WC, whole cell. B, immunostaining of p53 translocation in A2780/CP cells. A2780/CP cells were treated with 10 μM AT101 for 24 h. Cells were fixed and immunostained for p53 translocation as described under “Experimental Procedures.” The mitochondria were fluorescently stained with 50 nM MitoTracker. Nuclei were counterstained with DAPI. The arrows indicate the p53 mitochondrial translocation. Representative results of three experiments with consistent results are shown.
month of treatment or rashes earlier in the course of treatment (32). Thus, AT101 is clinically safe (10, 11) and could be used as a potential inducer of apoptosis in cancer treatment. Recent studies have revealed that gossypol can neutralize antiapoptotic Bcl-2 proteins and induce Bax activation (9, 33). However, the function of gossypol is not limited to effects on the interaction between anti- and proapoptotic Bcl-2 proteins. Some studies have demonstrated that gossypol could down-regulate Bcl-2, Bcl-xL, and XIAP expression (12) or induce Puma and Noxa expression (9). Therefore, understanding the mechanism by which AT101 induces apoptosis may identify potential targets for cancer therapies.

In this study, we demonstrated that AT101 induced Bax activation during apoptosis of chemoresistant ovarian cancer cells. However, we found no obvious changes in the expression levels of Bcl-2 family proteins in apoptosis. Further experiments indicated that reduction of Bax decreased but did not completely inhibit caspase-3 activation. Bax knockdown had no effect on Smac release or the Smac-XIAP interaction.

Our group and others have demonstrated that Bax resides in the cytosol of resting cells in an inactive state and, in response to various stimuli, undergoes specific conformational changes that allow its targeting and insertion into the mitochondrial outer membrane, where it forms a pore that allows the release of proapoptotic factors, such as cyt c and Smac, into the cytosol (20, 34, 35). A recent study demonstrated that p53 could accumulate in mitochondria and induce Smac release in Bax knockout cells (36). The Panton-Valentine leukocidin cytotoxin was directly targeted to mitochondria and triggered Smac release without Bax activation (37). However, it is unclear whether Smac release is independent of Bax activation in apoptosis. Our results show that Bax activation had no effect on mitochondrial Smac release during apoptosis of Bax WT cells. These results also suggest that other factors are involved in regulating Smac release in AT101-induced apoptosis. Previous experiments demonstrated that the Akt-p53 pathway mediated mitochondrial Smac release in AT101-induced apoptosis.

Previous research demonstrated the importance of Smac activation in apoptosis. Smac could induce or amplify the release of some mitochondrial apoptogenic proteins, such as cyt c or AIF, as well as the activation of caspase, even in Bax-deficient cells (25). Moreover, Smac mediated the apoptotic...
function of Puma by regulating Puma-induced mitochondrial events (24), and Bax-dependent release of Smac from mitochondria mediated the contribution of the mitochondrial pathway to death receptor-mediated apoptosis (23). These studies reveal that Smac is a major determinant in mitochondrial pathway-mediated apoptosis and that Smac can mediate the activation of the mitochondrial apoptogenic proteins. However, it is unclear whether Smac can mediate the activation of Bax, an important mitochondrial apoptogenic factor, in apoptosis. Our data provide evidence that Smac inhibition can decrease the Bax conformational change, translocation from cytosol to mitochondria, and oligomerization. Thus, Smac release is the upstream event of Bax activation and mediates Bax-induced apoptosis in AT101-treated cells. Indeed, overexpression of Smac enhanced cyt c release and caspase-3 cleavage even under Bax-deficient conditions.

A recent report revealed that Smac can induce caspase activation, and a study by another group suggested the existence of a caspase-mediated feedback amplification loop regulating cyt c release and Bak activation (24, 38). It is possible that Smac participates in this feedback amplification loop by providing the initial caspase activity to unleash further mitochondrial changes (such as Bax activation and cyt c release) that are necessary for full execution of apoptotic events. Indeed, our data indicate that XIAP overexpression attenuates Bax activation and caspase-3 cleavage. Studies are currently under way in our laboratory to elucidate the precise mechanisms by which Smac influences Bax activation.

Our results reveal that Smac release is a major determinant in AT101-induced apoptosis. Therefore, we can explain why Smac reduction inhibits caspase-3 cleavage, whereas Bax knockdown only partially prevents caspase-3 activation. Smac can disrupt caspase inhibition by IAP proteins to directly activate caspase-3 (13). The inhibition of Smac not only blocks the direct function of Smac on caspase-3 but also impedes Bax-mediated caspase-3 activation. In contrast, Bax reduction only inhibits Bax-mediated caspase-3 cleavage. Our findings also explain why AT101 can induce cell apoptosis in chemoresistant ovarian cancer cells. In chemoresistant cells, overexpression of Bcl-2 and Bcl-xl prevents or decreases Bax activation (8, 39, 40), whereas AT101 induces Smac release to enhance Bax activation, in addition to directly affecting Bax activation in chemoresistant cells. Therefore, the dual effects of AT101 are sufficient to initiate apoptosis in chemoresistant cells.

In summary, we explain the molecular mechanism of AT101 in ovarian cancer cells and provide the first evidence that Smac-mediated Bax activation is an important factor for AT101-induced apoptosis in cisplatin-resistant ovarian cancer cells. A thorough understanding of how AT101 works in cells may improve treatment outcomes for human ovarian cancer.

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