Triapine (3-aninopyridine-2-carboxaldehyde thiosemicarbazone) Induces Apoptosis in Ovarian Cancer Cells

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Journal of the Society for Gynecologic Investigation 2006 13: 145
DOI: 10.1016/j.jsgi.2005.11.004

Triapine (3-aminopyridine-2-carboxaldehyde thiosemicarbazone) Induces Apoptosis in Ovarian Cancer Cells

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OBJECTIVES: *Triapine (Vion Pharmaceuticals, New Haven, CT) is a potent ribonucleotide reductase inhibitor which exerts its antineoplastic acitivity by inhibiting DNA synthesis and repair. The objectives of this study were: (1) to determine whether Triapine has cytotoxic effects on epithelial ovarian cancer (EOC) cells; (2) to characterize the apoptotic cascade induced in response to this agent; and (3) to determine its utility in combination treatment with carboplatin and paclitaxel.*

METHODS: *Five EOC cell lines were treated with tenfold dilutions of Triapine (0.1 to 100 μM) for 24 and 48 hours. Cell viability was determined by the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega Corp, Madison, WI) and the morphologic features of apoptosis were observed using Hoechst staining. The apoptotic cascade was characterized by Western blot analyses.*

RESULTS: *All EOC cell lines treated with Triapine showed decreased cell viability in a time- and dose-dependent manner. Hoechst staining revealed nuclear shrinkage and chromatin condensation and fragmentation, which correlated with the occurrence of apoptosis. Western blots demonstrated that Bid activation was one of the initiating signals involved in the cascade. In addition, cleavage of XIAP and down-regulation of Akt were observed. We also demonstrated that Triapine enhances the cytotoxic effects of carboplatin and paclitaxel.*

CONCLUSIONS: *The present findings demonstrate that Triapine induces cell death through the induction of apoptosis. The initial activation of Bid indicates the involvement of the mitochondrial pathway. The demonstration that Triapine is an effective addition to a carboplatin regimen suggests the possibility of a new combination therapy for ovarian cancer. (J Soc Gynecol Investig 2006;13:145–52)*

KEY WORDS: Triapine, apoptosis, caspase, ovarian cancer.

Epithelial ovarian cancer (EOC) is the fourth leading cause of cancer-related deaths in women in the United States and is the most lethal of the gynecologic malignancies.[1] One of the major limitations in the treatment of EOC is the development of cross-resistance to a wide range of chemotherapeutic agents. Indeed, although 80% to 90% of patients initially respond to first-line chemotherapy such as carboplatin and paclitaxel, less than 10% to 15% will remain in remission.[2] Treatment advances have led to improved 5-year survival, approaching 45%; however, no advances have been made in overall survival. Therefore, the identification of new treatment regimen for EOC will be beneficial especially in patients with recurrent disease.

A novel agent that has been shown to induce cell death in a variety of tumor cell lines is 3-aminopyridine-2-carboxaldehyde thiosemicarbazone (Triapine; Vion Pharmaceuticals, New Haven, CT). Triapine is a potent ribonucleotide reductase (RR) inhibitor that is capable of interfering with DNA synthesis and repair.[3] Its effectiveness as a chemotherapeutic agent has been demonstrated in preclinical studies in both hematologic and solid tumors. Thus, in vitro, Triapine has been shown to inhibit the growth of murine L1210 leukemia and human KB nasopharyngeal carcinoma cell lines.[3] In vivo, it has been reported to inhibit the growth of the murine M109 lung carcinoma and the human A2780 ovarian carcinoma cell lines.[4] The results of phase I clinical trials in patients with leukemia and various solid tumors show that Triapine has relevant tumor inhibitory activity at the highest dose levels achieved and has an acceptable safety profile.[5–8]

It is now well established that the induction of apoptosis is a key mechanism by which chemotherapeutic agents like carboplatin and paclitaxel induce cell death.[9] Apoptosis is characterized by cell shrinkage, membrane blebbing, chromatin condensation, and nuclear fragmentation.[10] The molecular mechanisms by which anticancer drugs induce apoptosis are

From the Departments of Obstetrics and Gynecology and Pharmacology, Yale University School of Medicine, New Haven, Connecticut; and the Department of Obstetrics and Gynecology, Nanfang Hospital, The First Military Medical University, Guangzhou, China.

Supported by the Discovery to Cure Research Fund and Nicolas Brady.

The authors wish to thank Dr Anna Rice for help in the analysis of the combination treatments.

Address correspondence and reprint requests to: Dr Gil Mor, Department of Obstetrics and Gynecology, Yale University School of Medicine, P.O. Box 208063, 333 Cedar St., FMB 301, New Haven, CT 06520-8063. E-mail: gil.mor@yale.edu

1071-5576/06/$32.00
doi:10.1016/j.jsgi.2005.11.004

mediated by the death receptor-dependent and/or mitochondrial pathways.[11,12] The activation of either pathway leads to a cascade of caspases, which are specific proteases synthesized as zymogens and activated by cleavage.[10,13] Within these cascades, caspases can be divided into "initiator" or "effector" caspases. The initiator caspases, such as caspase-8 (death receptor-dependent pathway) and caspase-9 (mitochondrial pathway), mediate their oligomerization and auto-activation in response to upstream signals. The effector caspases, caspases-3, -6, and -7, cleave cellular substrates and precipitate apoptotic death.

In this study, we evaluated the effects of Triapine on three established EOC cell lines and two EOC cell lines isolated from malignant ovarian ascites. Our results show that Triapine induces apoptosis in all of the EOC cell lines tested and that short-term pretreatment with low-dose Triapine enhances the cytotoxic effects of carboplatin better than it enhances the effects of paclitaxel.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

Established human EOC cell lines SKOV3 (obtained from the American Type Culture Collection, Bethesda, MD), CP70 and A2780 (gifts from Dr T.C. Hamilton)[14] were propagated in RPMI-1640 medium (Sigma Chemical Co, St. Louis, MO) supplemented with 10% fetal bovine serum (Gemini Bioproducts, Woodland, CA), 1000 U/mL penicillin, 100 μg/mL streptomycin, 10 mM HEPES, 100 nM nonessential amino acids, and 1 mM sodium pyruvate (all from GIBCO, Carlsbad, CA). Primary human EOC cell lines R179 and R182 were isolated from freshly collected malignant ovarian ascites as previously described[15] and cultured in 50% 199 medium and 50% 105 medium (Sigma Chemical Co) supplemented with 15% fetal bovine serum, 4 ng/mL epidermal growth factor (Sigma Chemical Co), 1000 U/mL penicillin, 100 μg/mL streptomycin, 10 mM HEPES, 100 nM nonessential amino acids, and 1 mM sodium pyruvate. All cultures were incubated under standard culture conditions at 37C in 5% CO_2.

Treatments and Cell Viability Assay

A total of 5×10^3 cells were plated in triplicate wells in a 100-μL volume per well of a 96-well microtiter plate (BD Biosciences/Pharmingen, San Diego, CA). The cells were grown to 70% confluence and then incubated in reduced-serum phenol-depleted Opti-MEM medium (GIBCO) for 4 hours prior to treatment. Triapine (Vion Pharmaceuticals, New Haven, CT) was added to the medium from 20 mM stock in dimethyl sulfoxide (DMSO) to give various final concentrations (0.1, 1, 10, and 100 μM). Following 24 and 48 hours treatment, cell viability was evaluated using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega Corp, Madison, WI) according to the manufacturer's instructions. Optical densities of the samples were measured at 490 nm using an automatic microplate reader (Model 550; Bio-Rad, Hercules, CA). The values from the treated cells were compared with the values generated from the untreated control (DMSO only) and reported as percent viability. Each experiment was done in triplicate.

For combination treatments with carboplatin and paclitaxel (both from Sigma Chemical Co), cells were incubated with 1 μM Triapine for 2 hours and then treated with increasing concentrations of either carboplatin (50 to 200 μg/mL) or paclitaxel (0.2 to 2 μM) for another 22 hours.

For experiments with caspase-8 and caspase-9 inhibitors, 20 μM of the specific caspase-8 inhibitor, Z-IETD-FMK or the specific caspase-9 inhibitor, Z-LEHD-FMK (both from BD Pharmingen) were added to cells 30 minutes prior to Triapine treatment. For experiments with the specific caspase-2 inhibitor, Z-VDVAD-FMK (R&D Systems, Minneapolis, MN), 25 μM of the inhibitor was added 1 hour prior to treatment with Triapine.

Hoechst Staining

Cells (1×10^5) were seeded in chamber slides (BD Falcon, Franklin Lakes, NJ) and treated with Triapine (10 μM) for 48 hours as described above. Subsequently, cells were incubated with 2 μg/mL Hoechst 33342 dye (Molecular Probes, Eugene, OR) for 15 minutes at room temperature and then visualized by fluorescent microscopy.

Preparation of Cell Lysates and Measurement of Protein Concentration

Cells (1×10^6) were plated in a 100-mm culture dish and treated with Triapine as described above. Protein was prepared as previously described.[15] Briefly, after drug treatment, cells were scraped, pelleted at $300 \times g$ for 10 minutes, resuspended in lysis buffer (1% NP40 and 0.1% sodium dodecyl sulfate [SDS] in phosphate-buffered saline [PBS]) and incubated on ice for 20 minutes. The cell lysate was centrifuged at 14,000 rpm using a C3i centrifuge (AC1.14 rotor; SOCIETE JOUAN, Winchester, VI), for 15 minutes at 4C. The supernatant was collected and stored at −40C until further use. A cocktail of protease inhibitors was added to the supernatant prior to storage. Protein concentration was determined by BSA Calibration Assay (Pierce, Rockford, IL).

Measurement of Caspase-3/7, Caspase-8, and Caspase-9 Activities

Caspase-3/7, -8, and -9 activities were measured using the Caspase-Glo 3/7, Caspase-Glo 8, and Caspase-Glo 9 assays (Promega Corp), respectively, according to the manufacturer's instructions. Briefly, 10 μg of protein in a 50 μL total volume was mixed with 50 μL of equilibrated Caspase-Glo reagents. After incubating at room temperature for 1 hour, luminescence was measured using a TD 20/20 Luminometer (Turner Designs, Sunnyvale, CA). Activity is reported as the fold increase relative to no-treatment controls.

Western Blot Analyses

Proteins (20 μg) were denatured in sample buffer (2.5% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.15 M Tris-

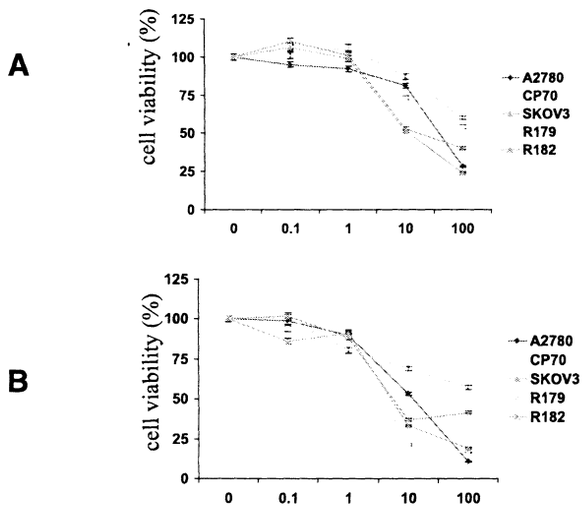

Figure 1. Triapine decreases the viability of EOC cells. The viability (in percentage, normalized to untreated cells) of EOC cells after treatment with increasing concentrations of Triapine for (A) 24 and (B) 48 hours. Data were compiled from at least three independent experiments, each done in triplicate. X axis: concentration μm.

HCl [pH 6.8], and 0.01% bromophenol blue) and subjected to 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to PVDF membranes (Immobilon; Millipore, Bedford, MA) at 100 V for 105 minutes. Subsequently, the membranes were blocked for 1 hour with 5% milk in PBS with 0.5% Tween-20 (PBS-T) and incubated overnight at 4C with primary antibody in PBS-T with 1% milk. The following antibodies and concentrations were used: 1:1000 mouse anti-caspase 8 (Oncogene Research Products, San Diego, CA), 1:5,000 mouse anti-caspase 9 (R&D Systems), 1:2000 mouse anti-Bcl-2 (BD Pharmingen), 1:5000 rabbit anti-Bid (Cell Signaling Technology, Inc, Beverly, MA), 1:500 mouse anti-Bax (BD Pharmingen), 1:1000 mouse anti-XIAP (BD Pharmingen), 1:1,000 rabbit anti-phosphorylated Akt (Cell Signaling Technology), 1:2000 rabbit anti-total Akt (Cell Signaling Technology), and 1:10,000 rabbit anti-actin (Sigma Chemical Co). After three washes with PBS-T, membranes were incubated for 1 hour at room temperature in 1:10,000 horse anti-mouse or goat anti-rabbit, both horseradish peroxidase–labeled (Vector, Burlingame, CA). Finally, proteins were visualized using enhanced chemiluminescence (Pierce Biotechnology).

RESULTS

Triapine Induces Cell Death in Chemo-resistant EOC Cells

To determine the effects of Triapine on EOC cells, three established EOC cell lines (CP70, A2780, SKOV3) and two EOC cell lines isolated from malignant ovarian ascites (R179, R182) were treated with tenfold dilutions of Triapine (0.1 to 100 μM) for 24 and 48 hours and cell viability was determined using the CellTiter 96 AQueous One Solution Cell Proliferation Assay. Triapine effectively decreased cell viability in a time- and dose-dependent manner in all of the EOC cell lines tested (Figure 1A-B). The most prominent effect on cell viability was observed following 48 hours of treatment, when four of five cell lines tested had IC_{50} values of $\leq$10 μM. Under these conditions, the most sensitive cell line was CP70 (IC_{50} ~5 μM) and the most resistant cell line was R179 (IC_{50} >100 μM).

Triapine Induces Apoptosis in EOC Cells

To test whether the decrease in cell viability observed after treatment with Triapine is due to apoptosis, EOC cells were stained with Hoechst 33342 dye after exposure to the drug. The dye stains condensed chromatin of apoptotic cells more brightly than chromatin of normal cells. As shown in Figure 2A, Hoechst staining, which correlates with the presence of cells with typical apoptotic nuclear morphology (nuclear shrinkage, DNA condensation and fragmentation), was present in the Triapine-treated cells (Figure 2A, panel D), but not in the nontreated controls (Figure 2A, panel C). To further confirm the activation of the apoptotic pathway, we evaluated the activity of the central effector caspase, caspase-3, using the Caspase-Glo 3/7 assay. Although not all of the cell lines responded equally, a significant increase in caspase- 3/7 activity was observed in all EOC cell lines tested following Triapine

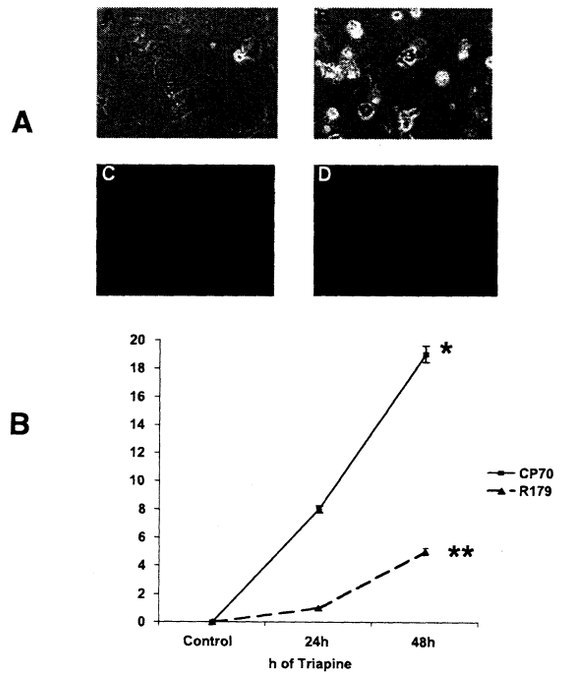

Figure 2. Triapine induces apoptosis in EOC cells. (A) Phase-contrast (A, B) and Hoechst 33342 dye staining of apoptotic nuclei (C, D) of untreated control (A, C) and Triapine-treated CP70 cells (B, D). (B) Caspase-3/7 activity of untreated control and Triapine-treated CP70 and R179 cells. Y axis: caspase-3/7 activity fold increase from control. *P = .0002, **P = .0004, both relative to untreated controls. Values represent the mean from three independent experiments.

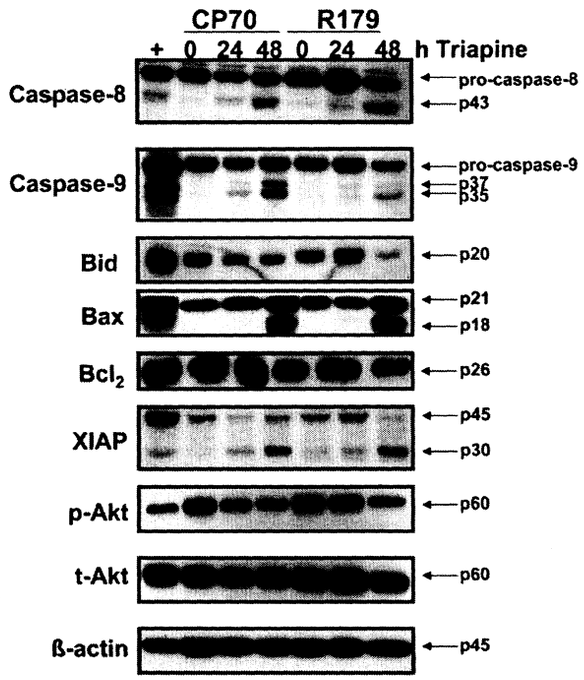

Figure 3. Characterization of the apoptotic cascade induced by Triapine. Western blot analyses showing the activation status of pro- and anti-apoptotic proteins in EOC cells after treatment with 10 μM Triapine. Results for CP70 and R179 cells are shown. Similar results were obtained with other EOC cell lines.

treatment. Figure 2B shows the results for CP70 (most sensitive to Triapine) and R179 (most resistant).

Triapine-Induced Apoptosis Involves Activation of the Mitochondrial Pathway

To characterize the components of the apoptotic cascade induced by Triapine, we first evaluated the activation of the initiator caspases, caspase-9 (mitochondrial pathway) and caspase-8 (receptor-mediated pathway) by Western blot analyses. The expression of the active forms of caspase-9 (p37 and p35) and the active form of caspase-8 (p43) increased in a time-dependent manner (Figure 3) following Triapine treatment. The active form of caspase-9 was visibly observed 48 hours post-treatment in the most sensitive cell line, CP70, and to a lesser extent in the most resistant cell line, R179. On the other hand, significant levels of the active forms of caspase-8 were present 48 hours post-treatment in both cell lines.

To establish whether the mitochondrial pathway is involved in Triapine-induced apoptosis, we determined the activation status of the pro-apoptotic mitochondrial proteins Bid and Bax and the anti-apoptotic protein Bcl_2. As shown in Figure 3, a decrease in the pro-form of Bid (p20), and therefore its activation, occurred 24 hours post-treatment in the most sensitive cell line, CP70, and 48 hours post-treatment in the most resistant cell line, R179. The cleavage of Bax occurred 48 hours post-treatment in both cell lines. No changes in Bcl_2 levels were observed.

Triapine Induces Cleavage of XIAP and a Decrease in Akt

Previously we and others have shown that the expression of XIAP correlates with chemo-resistance.[16–18] Similarly, Akt is an anti-apoptotic protein, which promotes survival of cells by preventing the activation of pro-apoptotic proteins such as Bax[19] and caspase-9[20] and by stabilizing XIAP.[21] To determine the effects of Triapine on these pro-survival proteins, Western blots were performed following treatment with Triapine. As shown in Figure 3, Triapine induced cleavage of XIAP and decreased the phosphorylated (p-Akt) form of Akt beginning 24 hours post-treatment in CP70 and 48 hours post-treatment in R179. The cleavage of XIAP and decrease in Akt therefore occurred prior to the significant appearance of the active forms of caspases-8 and -9.

Triapine-Induced Bid Activation Is Independent of Caspase-8

Since activation of the pro-apoptotic protein Bid was one of the earliest events observed, we hypothesized that Bid is one of the upstream signals in the Triapine-induced apoptotic cascade. Thus, to further characterize the steps in the apoptotic pathway induced by Triapine, we next sought to determine the signals upstream of Bid. Bid has been previously shown to be activated directly by caspase-8[22] and since our results showed low levels of the active form of caspase-8 at the same time Bid was found to be activated (24 hours post-treatment in CP70 and 48 hours post-treatment in R179; Figure 3), we next determined whether the observed Bid activation was caspase-8–dependent. To accomplish this, EOC cells were treated with Triapine in the presence or absence of the specific caspase-8 inhibitor, Z-IETD-FMK. The results show that even in the presence of the caspase-8 inhibitor, which produced significant inhibition of caspase-8 (Figure 4B), Bid was still activated (Figure 4A).

Triapine-Induced Bid Activation Occurs Upstream of Caspase-9

Since Bid can also be activated by caspase-3 as a mitochondrial feedback loop after cytochrome c release and caspase-9 activation, we next determined whether the activation of Bid was dependent on the activation of caspase-9. Thus, cells were treated with Triapine in the presence and absence of the specific caspase-9 inhibitor, Z-LEHD-FMK. Even though caspase-9 activation was significantly inhibited in the presence of Z-LEHD-FMK (Figure 4C), similar to results obtained with the caspase-8 inhibitor, Bid was still activated (Figure 4A).

Triapine-Induced Bid Activation Is Independent of Caspase-2

Another known activator of Bid is caspase-2.[23,24] To determine if Triapine-induced Bid activation is dependent on caspase-2, EOC cells were treated with Triapine in the presence and absence of the specific caspase-2 inhibitor Z-VD-VAD-FMK. In a manner analogous to the results obtained

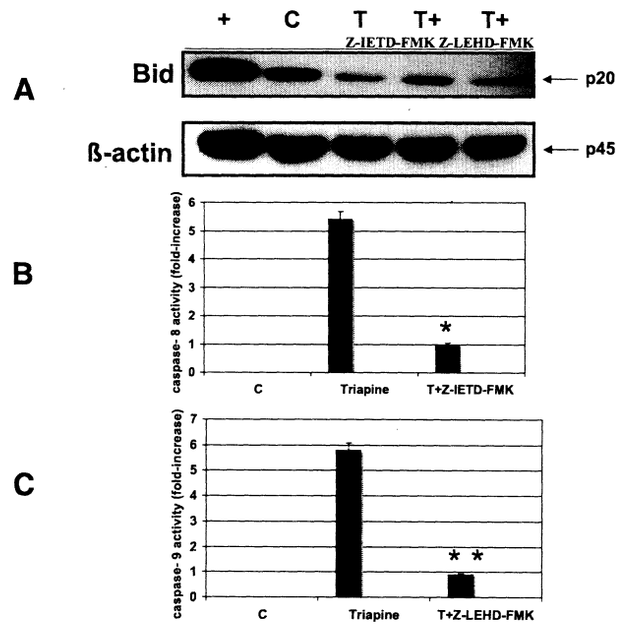

Figure 4. Triapine-induced Bid activation is both caspase-8 and caspase-9 independent. EOC cells were treated with 10 μM Triapine in the presence or absence of the specific caspase-8 inhibitor, Z-IETD-FMK, or the specific caspase-9 inhbitor, Z-LEHD-FMK. (A) Activation status of Bid was determined by Western blot analysis. Activity of caspases-8 (B) and -9 (C) was measured using the Caspase-Glo 8 and 9 assays, respectively. Results for CP70 cells are shown. *C*, no treatment control; *T*, Triapine-treated. $^{*}P = 5.3 \times 10^{-5}$, $^{**}P = .0003$.

with the inhibitors of caspase-8 and -9, Triapine-induced Bid activation was independent of caspase-2 (Figure 5).

Triapine Enhances the Cytotoxic Effects of Carboplatin and Paclitaxel in Ovarian Cancer Cells

The development of broad-spectrum chemo-resistance is a major problem in the treatment of ovarian cancer. Therefore, in the management of ovarian cancer patients, especially those in recurrence, the preferred approach is combination chemotherapy. Chemo-sensitizers that restore sensitivity to otherwise resistant cells or novel drugs that can enhance the effects of conventional agents theoretically can provide new modalities

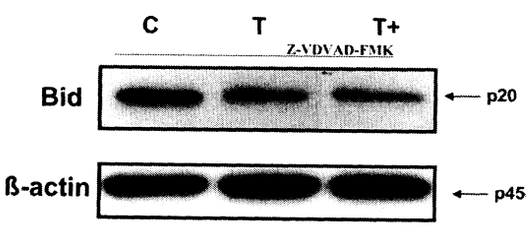

Figure 5. Triapine-induced Bid activation is caspase-2 independent. EOC cells were treated with 10 μM Triapine in the presence and absence of the specific caspase-2 inhibitor, Z-VDVAD-FMK, and the activation status of Bid was determined by Western blot analyses. Representative blots for CP70 cells are shown. *C*, no treatment control; *T*, Triapine-treated.

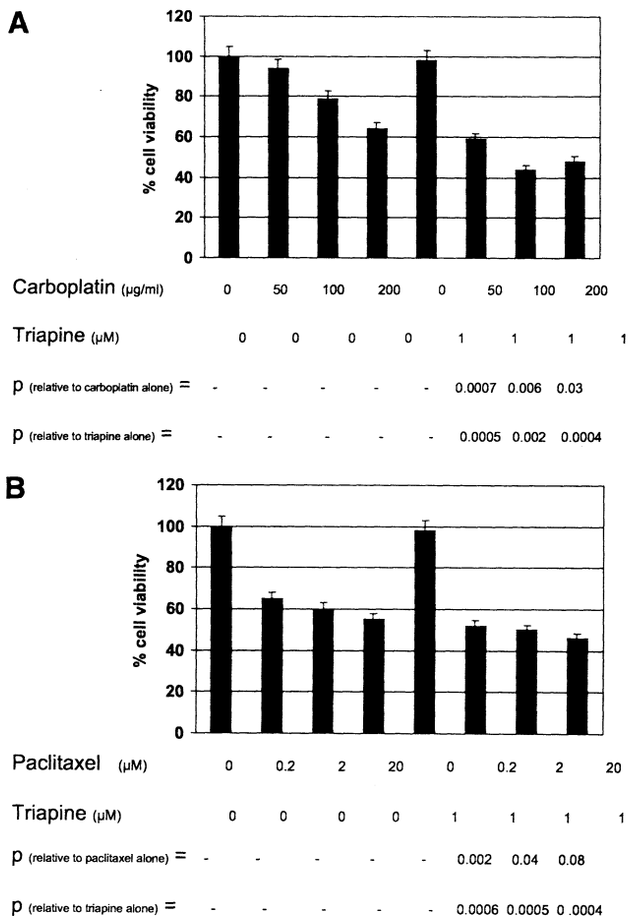

Figure 6. Triapine enhances the cytotoxic effect of carboplatin and paclitaxel. (A) EOC cells were treated with increasing concentrations of carboplatin and 1 μM Triapine for 24 hours or pretreated with 1 μM Triapine for 2 hours followed by increasing concentrations of carboplatin for another 22 hours. (B) EOC cells were treated with increasing concentrations of paclitaxel and 1 μM Triapine for 24 hours, or pretreated with 1 μM Triapine for 2 hours followed by increasing concentrations of paclitaxel for another 22 hours. Cell viability was determined using the CellTiter 96 AQueous One Solution Cell Proliferation Assay. The figure shows the results for CP70 cells.

of treatment. For these reasons, we ascertained whether a non-inhibitory level of Triapine had the ability to enhance the cytotoxic activities of the first line agents, carboplatin and paclitaxel, to EOC cells. To accomplish this, we pretreated EOC cells with a relatively low dose of Triapine (1 μM) for a short period of time (2 hours) before exposure to increasing concentrations of either carboplatin (50 to 200 μg/mL) or paclitaxel (0.2 to 2 μM) for an additional 22 hours. Triapine was removed from the medium after the 2 hour pretreatment and therefore was not present in the medium simultaneous with carboplatin or paclitaxel. Parallel exposures to carboplatin alone, paclitaxel alone, and Triapine alone were performed. The percentage of viable cells was then determined using the CellTiter 96 AQueous One Solution Cell Proliferation Assay. The results for CP70 cells are shown in Figure 6A-B. Pretreat-

ment with Triapine significantly enhanced the cytotoxic effects of carboplatin. While 50 μg/mL of carboplatin alone resulted in 6% decrease in cell viability, pretreatment with 1 μM Triapine followed by 50 μg/mL carboplatin decreased cell viability by 41%. Further enhancement of cytotoxicity was observed with the combination of Triapine and higher doses of carboplatin (Figure 6A). Likewise, pretreatment with low levels of Triapine enhanced the cytotoxic effects of paclitaxel, although not of the same magnitude as that observed with carboplatin (Figure 6B). These effects were observed in all the EOC cell lines tested.

DISCUSSION

Triapine is a potent inhibitor of RR and has been shown to have broad antitumor activity both in vitro and in vivo.[3,4] Phase I clinical trials in patients with both hematologic and solid tumors showed that it can be given safely at doses that achieve cytotoxic plasma concentrations.[6–8] In the present study, we evaluated the cytotoxic effects of Triapine on EOC cells and characterized the mechanisms by which it induces cell death.

RR inhibitors inhibit DNA synthesis and repair by depleting particularly deoxyadenosine triphosphate (dATP),[3,4] the consequences of which are more deliterious to cancer cells which have a higher growth fraction than most normal cells. One of the ways neoplastic cells acquire prolonged survival and resistance to cytotoxic drugs is by increasing the threshold of DNA damage needed to cause cell death. Triapine does not predominantly induce DNA damage; however, it can pose a major stress to malignant cells by preventing DNA synthesis and repair of damage to DNA produced by other agents.

The induction of apoptosis is one of the key mechanisms by which cells die in response to different types of chemotherapeutic agents, including those that induce DNA damage or prevent its synthesis and repair.[9] In the present study, we have characterized the apoptotic pathway induced by Triapine in EOC cells, and demonstrated that: (1) it induces apoptosis in all of the EOC cell lines tested; (2) it activates the mitochondrial apoptotic pathway through Bid; and (3) short-term pretreatment with essentially non-inhibitory doses of Triapine enhances the effects of both carboplatin and paclitaxel in inducing the death of EOC cells.

The decrease in cell viability following exposure to Triapine was due to the induction of apoptosis as demonstrated by chromatin condensation and caspase activation. The two most described pathways of apoptosis induction are the death receptor and the mitochondrial pathways. To trace the steps in the Triapine-induced apoptotic cascade, we evaluated the activation of the two initiator caspases, caspase-8 (death receptor pathway) and caspase-9 (mitochondrial pathway). We showed that both caspases are activated significantly 48 hours post-treatment with Triapine. To further determine the involvement of the mitochondrial pathway in the initial activation of apoptosis, we also evaluated the activation of the Bcl-2 family members, Bcl-2, Bax, and Bid. Our results showed that Triapine-induced apoptosis involved the early activation of Bid and a later activation of Bax. These findings suggest that Bid is the initial activator of the pathway and that Bax functions as an amplifier of the signal. Further confirmation of the involvement of Bax in the amplification loop is demonstrated by the significant increase in caspase-8, caspase-9, and caspase-3 activity following Bax activation.

Bid has been shown to have a caspase cleavage site and is activated by caspase-8.[22] This pathway represents the link between the death receptor pathway and the mitochondrial pathway. Interestingly, that is not the case in ovarian cancer cells following Triapine treatment. Our results show that despite the inhibition of caspase-8, Bid was still activated in the presence of Triapine. These findings suggest that Bid activation is independent of the membranal receptor pathway and caspase-8 activation occurs downstream of Bid.

Since we observed low levels of the active form of caspase-9 at about the time of Bid activation (24 hours post-treatement in CP70 and 48 hours post-treatment in R179), we sought to determine the spatial relationship between Bid and caspase-9. Our results indicate that Bid activation occurs prior to caspase-9, suggesting that Bid is the initial factor inducing activation of the mitochondrial pathway.

Although caspase-2 has been suggested to activate Bid in response to DNA damage,[23,25] our results showed that it is not involved in Triapine-induced Bid activation. Western blot analyses showed that Triapine retained its ability to induce Bid activation, even in the presence of the caspase-2 inhibitor. Taken together, these results show that Bid is indeed one of the early events in the Triapine-induced apoptotic cascade and that its activation is independent of caspase-8, -9, and -2. Recently, it was reported that in response to DNA damage, an unidentified aspartate-specific protease may be required for Bid activation.[26] The identification of this factor would provide relevant information to understand the signal pathways linking DNA damage and the mitochondrial apoptotic pathway.

Our work and that of others have established the role of XIAP in the development of chemo-resistance. It has been shown that high levels of XIAP prevent apoptosis induced by cytotoxic drugs such as cisplatin,[16] docetaxel,[17] and paclitaxel (our unpublished data), and that its down-regulation or inhibition by RNA interference[17,18] or by specific compounds[27] can restore chemo-sensitivity. When we evaluated the effects of Triapine on XIAP we observed that it induces its cleavage. Interestingly, the cleavage of XIAP precedes the significant activation of caspases-8, -9, and -3.

Another interesting observation is that Triapine induces the down-regulation of p-Akt. The Akt pathway is a major survival pathway that results in the inhibition of apoptosis. Specifically, Akt induces the phosphorylation of pro-caspase-9[20] and Bax,[19] thereby inhibiting their ability to induce apoptosis. It also targets the forkhead-transcription factors involved in the regulation of pro-apoptotic responses.[28] The down-regulation of Akt therefore, together with the down-regulation of XIAP, shifts the balance away from survival and towards cell death.

We, therefore, propose that the apoptotic pathway induced by Triapine involves the initial activation of Bid and the

mitochondrial pathway resulting in the release of cytochrome c and activation of caspase-9. In addition, the mitochondial proteins Smac/DIABLO and Omi are released from the mitochondria inducing the inhibition and cleavage of XIAP, respectively.[29–32] Activated caspase-9 then activates caspase-3, which then activates caspase-8. The concomitant decrease in full-length XIAP, as a result of cleavage, allows the full activation of the caspase cascade. Similarly, the decrease in XIAP results in the down-regulation of Akt as previously described.[33] Eventually, Bax is activated and amplifies the caspase cascade.

Recurrent ovarian cancer is one of the most difficult cancers to treat due to the widespread occurrence of chemo-resistance. In our laboratory, we have great interest in novel drugs that can not only induce apoptosis in EOC cells when given as monotherapy, but also those that can sensitize EOC cells or enhance the response of EOC cells to conventional first-line chemotherapeutic agents like carboplatin and paclitaxel. We have shown that pretreatment with low-dose Triapine can significantly enhance the cytotoxic effects of carboplatin and to a lesser extent paclitaxel. This differential effect may be explained by the mechanism of action of each agent: Triapine depletes the dATP pool in cells and carboplatin is a DNA-damaging agent, while paclitaxel induces the stabilization of microtubules. Therefore, in cells pretreated with Triapine, treatment with carboplatin will result in DNA damage that may not be repaired because of the low supply of nucleotides. Triapine and carboplatin therefore augment each other's effect. In contrast, paclitaxel does not induce DNA damage and therefore Triapine does not enhance its cytotoxic effects. The demonstration that the combination of Triapine–carboplatin induces significant cell death of EOC cells in vitro may initiate the development of new treatment protocols for ovarian cancer.

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