RESEARCH ARTICLE

Analysis of epothilone B-induced cell death in normal ovarian cells

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Abstract

We have investigated the mode of cell death induced by a new microtubule-stabilizing agent, epothilone B (EpoB, patupilone), and a clinically used medicine, paclitaxel (PTX), in normal ovarian cells. Using fluorescence microscopy, polyacrylamide gel electrophoresis preceding Western blot analysis, as well as spectrofluorimetric and colorimetric detection, we demonstrate that, compared to EpoB, PTX induced high time-dependent morphological and biochemical changes typical of apoptosis. Induction of apoptosis followed an early increase in p53 levels. Apoptosis reached its maximum at 24–48 h. At the same time, there was a significant increase in caspase-9 and -3 activity and PARP fragmentation, which suggests that an intrinsic path was involved. Apoptosis in MM14 cells was increased more by PTX than EpoB, and also induced more necrosis responsible for inflammation (1.4-fold) than EpoB.

Keywords: apoptosis; caspases; epothilone B; paclitaxel; PARP

Introduction

Many cancer cells circumvent normal apoptotic mechanisms to prevent self-destruction. Solid tumors can be resistant to apoptosis in general and to chemotherapy-induced apoptosis in particular. Cancer cells are at least as intrinsically resistant to apoptosis as normal cells and are able to employ immune escape mechanisms. There are problems with ascertaining windows of opportunity to selectively kill cancer cells without killing the host tissues (Hannun, 1997; Kleffel and Schatton, 2013).

Epothilones are a new group of microtubule (MT)-stabilizing agents with antitumor activity in taxane-resistant models, due to being a poor substrate of the P-glycoprotein efflux pump (Lee et al., 2009). Taxanes are currently used in the treatment of ovarian cancer. However, the development of new taxanes and its formulations has not resolved the problem of primary and acquired resistance, which has driven the search for alternative agents, such as epothilones, to replace them in the early stages of treatment. To date, only Ixabepilone has been approved by the US Food and Drug Administration (FDA). EpoB is being evaluated in phase III clinical trials for ovarian and peritoneal cancer (Kaiser et al., 2013), and its analogs are currently being used in the treatment of metastatic breast cancer (Shahabi et al., 2010; Cortes and Vidal, 2012).

Epothilone B exerts higher potency than paclitaxel (6–25-fold) (Bollag et al., 1995). EpoB induces G2/M phase cell-cycle arrest (Mozzetti et al., 2012). Both drugs inhibit MT dynamic instability. Epothilones stabilize MTs in the same way as paclitaxel, but with slightly higher efficiency. They bind to the same site on β-tubulin as paclitaxel, and share one polar place at C7-OH, whereas the thiazole side-chain is associated with a different region of β-tubulin than the taxanes (Goodin, 2008). For this reasons, EpoB competitively inhibits the binding of PTX to MTs (Fojo and Menefee, 2007). PTX and EpoB can induce early ROS production in cancer cells (Alexandre et al., 2006; Rogalska et al., 2013). Another mechanism of action shared by both tested drugs is the induction of apoptosis, but the molecular mechanisms behind the cytostatic and cytotoxic effects of EpoB are still being intensively investigated (Vishnu et al., 2012; Jelinek et al., 2013). Furthermore, the mode of cell death induced by EpoB in normal ovarian cells remains unclear.

Gynecological oncologists are assessing the potential therapeutic role of patupilone in recurrent platinum- and taxane-resistant ovarian and endometrial cancer patient populations. Ovarian cancer patients frequently relapse after...
first-line treatment based on platinum–taxane doublets. Therefore, epothilones might represent a therapeutic alternative in this setting. The terminal half-life \((t_{1/2})\) of patupilone administered intravenously is approximately 6 h, and has a wide volume of distribution to all tissues, including the brain. Epothilone B may also affect tumor vascularity. Both in a mouse melanoma model and in an orthotopically transplanted mammary carcinoma model, the administration of a single intravenous dose of patupilone (4 mg/kg) led to a decrease in interstitial fluid pressure, a possible surrogate biomarker of response for several well-known targeted agents and cytotoxics (Willett et al., 2004; Taghian et al., 2005; Diaz-Padilla and Oza, 2011). Similar to taxanes, epothilones also have some side effects, for example, patupilone leads to diarrhea in 53% of the patients. Other adverse events include nausea, fatigue, and vomiting (Diaz-Padilla and Oza, 2011). The long-term efficacy of taxanes may be limited because of myelosuppression and peripheral neuropathy.

Similar to taxanes, epothilones are non-selective and are cytotoxic against cancer and normal cells alike. For this reason, it is important to investigate the effect of EpoB on normal cells. Thus, we have characterized pathways involved in the mechanism of EpoB action in normal ovarian cells. In parallel experiments, another drug belonging to the very well characterized group of taxanes, PTX, was used for comparison. Little is known of the in vitro effects of MT depolymerization inhibitors on normal ovarian cells, most studies dealing mainly with samples from patients. Our observations could help increase the efficacy of epothilones in ovarian cancer treatment.

**Materials and methods**

**Chemicals**

PTX was obtained from Sequoia Research Products (Pangbourne, United Kingdom). EpoB and trypsin-EDTA were purchased from Sigma (St. Louis, USA). The Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum (FBS) were supplied by Cambrex (Basel, Switzerland). Caspase-3 Activity Assays were from Calbiochem (San Diego, USA); a Caspase-9 Fluorometric Assay Kit, the Z-LEHD-FMK inhibitor, and an Annexin V-Biotin Apoptosis Detection Kit were supplied by BioVision, Inc. (Milpitas, CA, USA). A p53 (Total) InstantOne™ ELISA kit was supplied by eBioscience, Inc. (San Diego, USA). All other chemicals and solvents were of high analytical grade and were obtained from Sigma.

**Ovarian cell line culture**

MM14 (mouse normal ovarian cell line) was obtained from the American Type Culture Collection (Rockville, MD, USA). MM14 cells were grown as a monolayer in DMEM supplemented with 10% FBS, penicillin (10 U/mL), and streptomycin (50 μg/mL) under standard conditions: 37°C, 100% humidity, the atmosphere being 5% CO2 and 95% air. The cells were routinely screened for Mycoplasma contamination.

Petri dishes were used to prepare samples for double staining with the Hoechst 33258/PI method, Annexin V/PI assay \((3 \times 10^5 \text{ cells}/60 \text{ mm dish})\), and Western blotting analyses \((3 \times 10^6 \text{ cells}/100 \text{ mm dish})\). Petri dishes (100 mm) were also used for p53, caspase-3, and caspase-9 assays \((10^6 \text{ cells/dish})\). After 24 h (the time necessary to ensure that cells were in the exponential growth phase), the medium was refreshed. Drugs were added and the cells were incubated for 2–72 h, depending on the test. EpoB and PTX were dissolved in ethanol. The drugs were aliquoted in small portions (20–50 μL) each and stored frozen at −20°C. Concentrated drug solutions were thawed immediately before use, and added directly to the cell medium after dilution with PBS to the desired final concentration.

**MTT assay**

The cytotoxicity of the drugs to normal cells was estimated by the standard microplate MTT colorimetric method. Logarithmically growing cells \((25 \times 10^3 \text{ cells/well})\) were seeded on 96-well plates. After 24 h, drugs were added and the cells were incubated for 72 h. The medium was removed, and after two washes with PBS, 50 μL MTT (final concentration 0.5 mg/mL) was added to the cells. The microplates were incubated for 4 h at 37°C before the medium was aspirated from each well and the violet formazan crystals resulting from MTT reduction by metabolically viable cells dissolved in 100 μL of DMSO per well. Absorbance was measured at 570 nm with a microplate reader (Awareness Technology, Inc., USA) (Carmichael et al., 1987a, b). On the basis of the MTT assay, the IC50 parameter was determined by dividing the obtained absorbance of the drug-treated samples by that of control cells.

**Annexin V-FITC and propidium iodide labeling**

Plasma membrane changes characteristic of apoptosis were analyzed by double staining with Annexin V-FITC and PI using a flow cytometer (LSRII, BD Biosciences). The cells were cultured with drugs for 2–48 h. At certain times, the cells \((1 \times 10^6 \text{ cells/mL})\) were removed from the culture dishes and suspended in Annexin binding buffer. Annexin V–FITC and PI were added to the cell suspension, and the cells were incubated at room temperature for 10 min in the dark. Three populations of cells were observed: (1) viable cells: FITC negative, PI negative, (2) apoptotic: FITC positive, PI negative; and (3) necrotic: FITC positive, PI positive. Cell observations were made using Eclipse E-600 epifluorescence...
microscope (Nikon, Japan). All images were recorded at exactly the same integration time using a DS-F1i CCD camera (Nikon). Cells were also analyzed by flow cytometry (LSRII, BD Biosciences) using the FL2 channel.

Double staining with Hoechst 33258 and propidium iodide

To determine the ratio between live, apoptotic, and necrotic cells, simultaneous cell staining with Hoechst 33258 and PI was used. At chosen time-points from 2 to 48 h, cells were removed from culture dishes by trypsinization, centrifuged, and suspended in PBS at 1 × 10⁶ cells/mL. To 100 µL cell suspension, 1 µL Hoechst 33258 (0.13 mM) and 1 µL PI (0.23 mM) were added, and the cells were incubated at room temperature for 10 min in darkness. The cells were classified on the basis of their morphological and staining characteristics as: live (blue fluorescence), early apoptotic cells (intensive bright blue fluorescence), late apoptotic cells (blue-violet fluorescence), and necrotic (red fluorescence) (Gasiorowski et al., 2001). At least 300 cells were counted on each slide, and each experiment was done in triplicate. The percentages of particular cell types were determined from the total number of cells.

Caspase activity

The activity of caspase-3 and -9 was estimated with fluorimetric assay kits. Cells harvested at 4, 24, and 48 h of drug incubation were briefly lysed. Cell extracts were mixed with the reaction buffer and incubated with caspase substrates. Caspase-3 has a substrate specificity for the amino acid sequence Asp–Glu–Val–Asp (DEVD). The basis for the assay is the 7-amino-4-methylcoumarin-derived substrate Z-DEVD-AMC (where Z represents a benzyloxycarbonyl group). The fluorescence of AMC was measured at 440 nm after excitation at 360 nm. Caspase-9 recognizes the sequence Leu–Glu–His–Asp (LEHD). The intensity of fluorescence of the released 7-amino-4-trifluoromethyl coumarin (AFC) was analyzed using a Fluoroscan Ascent FL plate reader (Labsystem, Sweden). The excitation and emission wavelengths of AFC are 390 and 510 nm, respectively. To confirm that the fluorescent signal indicated the activity of the correct caspase, caspase-3 inhibitor (1 µL of 1 mM Ac-DEVD-CHO) or caspase-9 inhibitor (1 µL of 10 mM Z-LEHD-FMK) was added to the lysate prior to the addition of caspase substrate. The activity of caspase-3 and -9 was expressed as a ratio of the fluorescence of drug-treated samples relative to the corresponding untreated controls taken as 100%.

P53 level

After treating cells with the drugs (2–48 h), the cells were washed with PBS and lysed. Clarified cell extracts were added to triplicate wells for determining cellular p53 levels. Subsequently, we introduced an antibody cocktail containing capture, detection, and amplification antibodies for 1 h. The plates were washed with the wash buffer included in the kit. p53 protein was detected using TMB colorimetric substrate. The reaction was inhibited by adding a stop solution reagent generating a yellow color, which was recorded at 450 nm with a PowerWave, BioTek microplate reader.

Western blotting

The cells were lysed in extraction buffer containing protease inhibitor cocktail and PMSF (Invitrogen). Protein concentration was determined using the Bradford method. SDS–polyacrylamide gel electrophoresis (10%) and transfer of proteins (100 µg/lane) to nitrocellulose membranes were done by standard procedures. To confirm equal loading and transfer, a PageRuler prestained protein ladder (Fermentas, Inc.) was used. After blocking nonspecific sites, the membranes were incubated with rabbit monoclonal antibody against PARP-1 diluted 1/5,000 (anti-PARP-1, Millipore). Rabbit anti-GAPDH polyclonal antibody was used as internal controls diluted 1/200 (AbD Serotec). Signals were detected using an alkaline phosphatase-conjugated secondary antibody (goat anti-rabbit, Millipore) diluted 1/400 and a colorimetric detection system (Vector Laboratories, Inc.).

Statistical analysis

Data are expressed as mean ± SD. Analysis of variance (ANOVA) with the Tukey post hoc test was used for multiple comparisons. All statistics were calculated using the Statistica software (StatSoft, Tulsa, OK, USA), and P < 0.05 was considered significant.

Results

Epotiholone B induces a type of cell death that has characteristics of apoptosis

The cytotoxic activity of EpoB and PTX was determined by the MTT test. The IC₅₀ values were 70.5 nM and 1 µM for EpoB and PTX, respectively. To compare the apoptotic effects of both drugs, we used the IC₅₀ of the drugs in all tests.

The number of Annexin-positive cells for the tested drugs increased linearly over time. Annexin-positive cells were seen after 2 h of EpoB and PTX treatment. After 24 h incubation with EpoB, the fraction of Annexin-positive cells amounted to ~16% and increased to 27% at 48 h. Greater changes were observed in PTX-treated cells. The maximal increase in the number of Annexin-positive cells was found at 48 h (~43%). At this time, the level of necrotic cells increased significantly (24%) (Figures 1A and 1B). Significant differences between
EpoB and PTX in terms of Annexin-positive cell levels were recorded for all times of incubation (17, 31, 26, and 16%, respectively, for 2, 4, 24, and 48 h). PS externalization was monitored under a fluorescence microscope (Figure 2). Control cells stained negative for Annexin V-FITC (data not shown). EpoB and PTX treatment resulted in cells with strong Annexin V-positive fluorescence. The majority of cells were negative for propidium iodide, indicating that they were at an early stage of apoptosis. Collectively, these findings provide evidence that both EpoB and PTX induced cell death through apoptosis. Quantitative analysis of the fractions of viable, apoptotic, and necrotic cells is set out in Figure 3. Both drugs induce apoptosis and necrosis. After 2 h of incubation, early apoptotic cells were the predominant fraction in cells treated with EpoB and PTX. Significantly greater time-dependent apoptotic changes were found for PTX (48 h, 15% early apoptotic cells), while after the same time EpoB led to only 4% apoptotic cells. After prolonged incubation, more cells in late apoptosis (mainly in EpoB-treated cells) and necrosis appeared. The greatest statistically

Figure 1 Flow cytometric analysis. Apoptosis in MM14 cells as measured using an Annexin V-FITC apoptosis detection kit. (A) Phosphatidylserine (PS) externalization in the MM14 cell line after incubation with IC\textsubscript{50} of EpoB or PTX for 48 h. The histograms show viable, apoptotic, and necrotic cell fractions. (B) Quantitative results of the effects of the drugs on apoptotic and necrotic cell levels. A representative experiment out of four conducted is shown. (*) Statistically significant differences relative to control cells, taken as 100%, \( P < 0.05; (#) \) statistically significant differences between samples incubated with EpoB or PTX, \( P < 0.05. \)
significant changes were recorded for treatment with PTX (after 48 h, 21% of the cells being dead), and the lowest for EpoB (after 48 h, 13% of the cells being dead).

**Activation of caspases**

Changes in the levels of activity of caspase-3 and -9 after treatment with EpoB and PTX were determined with the use of prepared sets containing substrates for caspase-3 and -9. The activity of the analyzed cysteine proteases was measured following determination of protein concentration in the tested samples. The final results were expressed as a percentage of the activity of a given cysteine protease, where the fluorescence of the control (not treated with the drug) was taken as 100%. Experiments were conducted simultaneously with inhibitors of the activity of caspase-3 (Ac-DEVD-CHO) and -9 (Z-LEHD-FMK) to ascertain that these were the enzymes activated by EpoB or PTX. The changes at 24 h of drug treatment were 165% for EpoB and 334% for PTX compared to the control (100%). After a prolonged incubation, EpoB gave a caspase-9 activity of 121% of the control, while the activity of PTX-treated cells had returned to the control. The caspase-9 inhibitor (Z-LEHD-FMK) decreased enzyme activity both in cells treated with PTX and EpoB (Figure 4). The downstream effector protease, caspase-3, was activated upon drug treatment (Figure 5), which was dependent on the time of incubation. EpoB significantly increased caspase-3 activity after 24 h (198%) and 48 h (158%). Greater changes in caspase-3 activity were observed after PTX incubation, with a maximum at 48 h (264%). In the presence of caspase-3 inhibitor, enzyme activity was decreased. EpoB induced 2.3-fold lower activity of caspase-3 and twofold lower activity of caspase-9 after 24 h of incubation compared to PTX.

**P53 assay**

EpoB induced an early (2 and 4 h incubation) increase in the level of p53 (123% of control). Prolonged treatment led to a decrease to 113%. With PTX, the increase in p53 levels came later (after 4 h) than with EpoB. At 48 h incubation with PTX, p53 levels returned to control values (Figure 6).

**PARP cleavage**

EpoB and PTX induce PARP activation in normal mouse ovarian cells (Figure 7). The highest level of caspase-3 activity was observed after 24 h of exposure, at which time 116 kDa PARP had been cleaved into signature 85 and 25 kDa fragments. During the course of EpoB and PTX treatment,
the loss of the Mr 116,000 polymerase was accompanied by
the appearance of an Mr \( \sim 25,000 \) polypeptide recognized by
this antibody. Similar expression was seen with both drugs.

Discussion

The mechanism of action of epothilones on normal cells is
very poorly studied, our knowledge of the cytotoxicity of this
class of drugs being derived mainly from cancer cell tests.

This article focuses on changes in normal ovarian cells under
the influence of EpoB.

Drug dose and metabolism, the organism from which the
cells are derived, and their doubling time are very important
factors in terms of cell response to EpoB treatment. We
previously determined the IC\(_{50}\) dose of EpoB for SKOV-3
cancer cells to be 27.5 nM (Rogalska et al., 2013). Normal
cells also die after treatment with cytotoxic drugs; we found
that normal ovarian cells are 2.6-fold more resistant to EpoB

Figure 3 Apoptosis detection. (A) Representative viable, early and late apoptotic, and necrotic cell fractions at different time points following the
treatment of MM14 cells with IC\(_{50}\) concentrations of EpoB and PTX. (B) The effect of EpoB and PTX on the levels of early and late apoptotic and necrotic
cells. Results are presented as means \( \pm \) SD of four experiments.
samples incubated with EpoB or PTX, treated with an IC50 dose of EpoB and PTX for 48 h in the presence or absence of a caspase-9 inhibitor (Z-LEHD-FMK, i9). Results are presented as mean ± SD of four independent experiments. (*) Statistically significant differences relative to control cells, P < 0.05; (+) statistically significant differences between samples incubated with caspase-9 inhibitor and with EpoB or PTX, P < 0.05; (#) statistically significant differences between samples incubated with EpoB or PTX, P < 0.05.

Figure 4 Changes in caspase-9 activity in MM14 cells. Cells were treated with an IC50 dose of EpoB and PTX for 4–48 h in the presence or absence of a caspase-9 inhibitor (Z-LEHD-FMK, i9). Results are presented as mean ± SD of four independent experiments. (*) Statistically significant differences relative to control cells, P < 0.05; (+) statistically significant differences between samples incubated with caspase-9 inhibitor and with EpoB or PTX, P < 0.05; (#) statistically significant differences between samples incubated with EpoB or PTX, P < 0.05.

Figure 5 Changes in caspase-3 activity in MM14 cells. Cells were treated with an IC50 dose of EpoB and PTX for 4–48 h in the presence or absence of a caspase-3 inhibitor (Ac-DEVD-CHO, i3). Results are presented as mean ± SD of four independent experiments. (*) Statistically significant differences relative to control cells, P < 0.05; (+) statistically significant differences between samples incubated with caspase-3 inhibitor and with EpoB or PTX, P < 0.05; (#) statistically significant differences between samples incubated with EpoB or PTX, P < 0.05.

EpoB cytotoxicity in MM14 cells

than the previously tested cancer cells (Rogalska et al., 2013). One of the main causes of tumor cell resistance to drugs involves membrane transporters. Cancer cells have a higher expression of ABC proteins, which remove cytostatics, than normal cells (Pasquier et al., 2011). It is difficult to compare the in vitro toxicity of drugs to normal ovarian cells with their in vivo effects (Sundman-Engberg et al., 1998). However, Park et al. (1980) showed a higher probability of complete responses in patients whose leukemic cells were more sensitive to cytostatic drugs than their normal bone marrow cells, which were tested simultaneously. On the other hand, Ajani et al. (1985) found doxorubicin was more toxic to normal bone marrow CFU-GM than to tumor cells grown in liquid culture.

The type of cell death is of importance, with apoptosis being of a non-inflammatory nature (Carrara et al., 2012). We found adverse morphological and biochemical changes occurred in a normal ovarian cell line after PTX stimulation, but fewer apoptotic cells were observed after treatment with EpoB than PTX. The number of apoptotic cells decreased with time of EpoB incubation, in contrast to PTX.

The greater apoptotic effect of PTX was confirmed by measuring phosphatidylserine (PS) externalization, which is an early marker of programmed cell death. There was a time-dependent increase in the percentage of apoptotic cells upon treatment with EpoB and PTX; however, PTX led to a 1.6-fold higher number of apoptotic cells. More necrotic cells were seen after treatment with PTX in comparison to EpoB (1.4-fold). Necrotic death causes loss of plasma membrane integrity is lost, and cytosolic content released from the cells leads to inflammatory or autoimmune responses. A markedly higher increase in apoptosis after 6 days of treatment with 100 nM PTX (210% vs. controls) has been reported in human umbilical vein endothelial cells (HUVEC) (Bocci et al., 2002). Similar results came from endothelial cells exposed to a low-dose regimen of PTX for 24 h. Significant inhibition of proliferation occurred only at the highest concentrations of the tested drug—cell hypersensitivity was induced using the continuous long-term (144 h) exposure protocol. A high dose of taxol can induce cell death in normal cells, while a low dose of taxol inhibits only fast-dividing tumor cells (George et al., 2009).

Detection of fragmented DNA and apoptotic cell bodies by acridine orange labeling suggests that this sequential mode of cell death occurs during EpoB treatment of glioblastoma cells (Quick, 2008). P53 plays a critical role in apoptosis of irreversibly damaged cells. Being a tumor suppressor, it is associated with the molecular mechanism of chemotherapy resistance. p53 localizes to MTs and, in response to DNA damage, is transported to the nucleus via the MT minus-end-directed motor protein, dynein. We found that EpoB increases the level of p53 earlier than PTX, and its level returns to the control value in 24 h. The level of p53 after PTX treatment continued to be high, which can also explain the presence of apoptotic changes in PTX-treated cells after longer time intervals. Treatment with low concentrations of PTX (3 and 6 nM), EpoA or EpoB (3 nM) does not disrupt the MT network, but suppresses MT dynamics and enhances p53 nuclear accumulation, leading to the activation of the p53-downstream target genes of apoptosis (Giannakakou et al., 2002). Mutational analyses revealed increased response rates in models with mutations in the p53 gene or low p53 mRNA levels. Tumors with wild-type p53, as well as with high
expression of genes involved in cell adhesion/angiogenesis, are more likely to be resistant to sagopilone (SAG) therapy (Hammer et al., 2010). Moreover, epothilones have been used in clinical trials on prostate cancer. Loss of the p53 function increases the sensitivity of cells to PTX, and epothilone is more active against transformed prostate epithelial cells with mutant p53 than wild type p53 (Ioffe et al., 2004). A clinically achievable concentration of epothilone B induced a cytotoxic response in p53 mutant glioblastoma cells as a consequence of surviving downregulation and tubulin redistribution, while a cytostatic response was observed in p53 null glioblastoma cells with a modest increase in surviving expression post-epothilone B treatment. The author suggested that EpoB is a potential alternative to classic MT inhibiting agents (i.e., vincristine, paclitaxel) used to treat glioblastomas with p53 mutations (Quick, 2008). The dosage of the drug and the type of cancer determines its mechanism of action. Treatment of A549 cells with a low concentration (2.5 nM) of SAG upregulated the direct transcriptional target genes of p53, such as CDKN1A, MDM2, GADD45A, and FAS. Knockdown of p53, which inhibits the transcriptional induction of p53 target genes, led to a significant increase in apoptosis induction in A549 cells treated with a low concentration of SAG. Similar results have been obtained using a higher concentration of SAG (40 nM) (Winsel et al., 2011). On the other hand, p53 had only a moderate effect on SAG-induced cell death in HCT 116 colorectal cancer cells (Hoffmann et al., 2008).

The significance of apoptotic cell death in response to chemotherapy has become increasingly important. Apoptotic protease-activating factor-1 (APAF-1) activates the initiator caspase-9, which leads to the activation of the executioner caspase-3. In determining whether caspases are involved in EpoB-mediated apoptosis in normal cells, we found higher levels of caspase-9 and -3 in cells with PTX. Activated caspases have also been detected in some normal cells, such as HUVEC (Long et al., 2012), normal human primary keratinocytes (NHEK) (Szmyd et al., 2012), and human macrophage cells (Boronkai et al., 2009), along with similar results in cancer cells. In HCT 116 colorectal cancer cells, caspases are activated through a canonical pathway which involves caspase-9 and -3 as a consequence of mitochondrial cytochrome c release (Hoffmann et al., 2008). After Fludelone (26-trifluoro-(E)-9,10-dehydro-12,13-desoxyepothilone B) treatment, caspase-3, -8, and -9 were activated in multiple myeloma (MM) cells. Immunoblots of whole-cell lysates showed typical 17 kDa products of caspase-3 or 85 kDa products of PARP cleavage accompanied by a decrease in the detection of the uncleaved form of caspase-3 or PARP. The activity of caspase-8 showed a 3- to 4-fold increase, whereas that of caspase-9 rose 8- to 10-fold relative to untreated controls (Wu et al., 2005).

In EpoB- and PTX-treated MM14 cells, we also observed poly-(ADPribose) polymerase (PARP) fragmentation. Similar results have been obtained for A549 cells, where the p85 cleaved epitope was detected as a marker of apoptosis. The results for PARP cleavage were confirmed by the caspase-3 activation assay and the sulforhodamine-B cell survival assay (Giannakakou et al., 2002).

Understanding the biochemical and molecular basis of cell death will make it possible to create a better therapeutic window for epothilone action while simultaneously preserving normal cells. Detection of apoptosis at early stages, before morphological changes occur, is critical for cell death. PTX-mediated translocation of PS from the inner face of the plasma membrane to its outer surface was higher than that mediated by EpoB. This process sends a signal to neighboring cells that a cell is ready to be phagocytosed. EpoB and PTX induced time-dependent apoptosis and necrosis. Apoptosis is the prevalent form of normal ovarian cell death during the first 24–48 h after drug treatment. The levels of early and late
apoptotic cells were evaluated quantitatively. By using double staining, we confirmed that PTX generates higher morphological changes such as nucleus shrinking, chromatin condensation, and apoptotic body formation than EpoB. Extended incubation time allowed for observing cell disintegration and a higher number of necrotic cells. Increased expression of p53, a crucial factor in apoptosis regulation, which mediates the induction of programmed cell death, was mostly observed following exposure to PTX. Assuming that the drugs lead to disturbances in mitochondria, the levels of the initiator caspase-9 were measured. The activation of caspase-9 suggests the formation of apoptosomes, making it possible to evaluate the level of the executive caspase-3, which triggers PARP cleavage. We do not exclude the possibility that an extrinsic pathway may contribute to apoptosis. Generally, normal ovarian cells are less sensitive to apoptotic stimulation by EpoB than by PTX, which translates into the degree of cell damage. We will continue to determine whether epothilones can be safe for normal ovarian cells and are efficient drugs with a clear mechanism of action for ovarian cancer treatment.

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