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Bcl-2 Down-Regulation Is a Novel Mechanism of Paclitaxel Resistance

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ABSTRACT

Taxanes act by inhibiting microtubule dynamics; in this study, we have investigated mitochondria as an additional target of taxanes. We incubated isolated mitochondria in the presence of taxanes with or without stimulation of the mitochondrial respiratory state. Results showed that they rapidly induced the loss of $\Delta\psi_m$ after stimulation of the respiratory state. To evaluate the binding of [^{14}C]paclitaxel to isolated mitochondria, mitochondrial proteins were precipitated yielding 18.6 ± 2.1 cpm/μg of protein. After stimulation of the respiratory state, binding of [^{14}C]paclitaxel increased up to 163.2 ± 46.7 cpm/μg of protein. CPM values after Bcl-2 immunoprecipitation was 62.8-fold higher than those of the control antibody, thereby indicating the involvement of Bcl-2 in paclitaxel binding. Then, we established a panel of A2780 cell lines resistant to increasing doses of paclitaxel alone or to high doses of paclitaxel/cyclosporin A (A2780 TC cells). In both cases, Bcl-2 expression was consistently down-regulated, whereas levels of other members of the Bcl-2 family, such as Bax and Bcl-x, did not change in paclitaxel-resistant cell lines. When A2780TC cells were stably transfected with a Bcl-2 construct, paclitaxel sensitivity was partially restored, thereby supporting a direct role of Bcl-2 down-regulation in the maintenance of drug-resistance. Finally, we examined Bcl-2 by immunohistochemistry in a small subset of ovarian cancer paclitaxel-resistant patients and we noticed that the protein is down-regulated in this clinical setting with respect to the expression levels found in drug-sensitive tumors. These findings demonstrate that Bcl-2 is an additional intracellular target of taxanes and that its down-regulation is involved in taxane resistance.

Taxanes are natural products derived from trees of the genus *Taxoidaceae*. The first taxane introduced in cancer therapy was paclitaxel, firstly isolated from *Taxus brevifolia* (Schiff et al., 1979). The clinical success of taxanes is dependent on the excellent response rate in second-line treatment of relapsing/resistant cancers and on the efficacy of taxanes in the multichemotherapeutic approach of ovarian and breast cancer (Verweij et al., 1994).

In earlier studies, the microtubule network appeared as the main target of paclitaxel (Schiff et al., 1979; Manfredi et al., 1982). In fact, taxanes bind to β-tubulin subunits, thereby disrupting normal turnover of the microtubules. The final consequence is the arrest of the cell cycle in M phase with formation of aberrant mitosis and the activation of cell death pathways (Jordan et al., 1993). Along with arrest in M phase of the cell cycle, taxanes have also been reported to induce post-translational serine phosphorylation of the Bcl-2 protein (Haldar et al., 1995). The *BCL2* gene is the homologous of the nematode CED-9 gene product (Hengartner and Horvitz, 1994) and is capable of prolonging cell survival by inhibiting apoptotic cell death. Overexpression of Bcl-2 has been observed in follicular lymphoma, where this protein is deregulated by chromosomal translocation, and in a large number of human tumors, including breast, lung, and prostate cancer.

Disagreement exists on the levels of Bcl-2 and resistance to taxanes. A strong suggestion for a direct role of Bcl-2 in mediating paclitaxel sensitivity comes from the observation that a cell line not expressing Bcl-2 is resistant to paclitaxel-induced apoptosis (Haldar et al., 1996). Further support for this view stems from the observation that paclitaxel is able to entrap, from a random peptide library, a panel of peptides showing a high degree of structural homology with the disordered loop of Bcl-2, thereby indicating the latter as a motif for direct paclitaxel binding (Rodi et al., 1999). In apparent contrast with this view, overexpression of exogenous Bcl-2 or Bcl-xL protected HL-60 leukemic cells from paclitaxel-in-

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ABBREVIATIONS: DMSO, dimethyl sulfoxide; MDR, multidrug resistance; P-gp, p-glycoprotein; $\Delta\Psi_m$, mitochondrial membrane potential; JC-1, 5,5′6,6′-tetraethylbenzimidazolcarbocyanine iodide; PCR, polymerase chain reaction; VDAC, voltage-dependent anionic channel; PT, mitochondrial permeability transition pore; wt, wild type; ANOVA, analysis of variance; RT, reverse transcription.

duced apoptosis (Tang et al., 1994; Ibrado et al., 1997), so the role of Bcl-2 as modulator of paclitaxel sensitivity remains controversial. In addition, in clinical studies, Bcl-2 up-regulation is sometimes related to a better clinical outcome; in other cases, however, it is a marker of poor prognosis (Blagosklonny, 2001). Such a paradox can be explained by taking into consideration Bcl-2 levels along with the functional status of the death machinery. In fact, Bcl-2 up-regulation can be a marker of still-functional death machinery with consequent maintained sensitivity to chemotherapy-induced apoptosis (Blagosklonny, 2001).

Several authors have pointed out the possibility that taxane-dependent antitumor activity could originate not only from its effects on microtubule assembly (Danesi et al., 1995; Moos and Fitzpatrick, 1998; Pae et al., 1998) but also through the interaction with intracellular targets other than microtubules, although conflicting data have been reported (Blagosklonny and Fojo, 1999). Nevertheless, this hypothesis still remains very attractive because natural products have a marked pleiotropism; during thousands of years of evolution, plants have performed "combinatorial chemistry" by modification of molecules with pre-existing biological activities to overcome the environmental selective pressure. Therefore, a new molecule originates from the framework of a compound with previous biological activities.

In this work, we approached this assumption and studied mitochondria as a possible target for taxanes. Several reports have indicated that taxanes can interact with isolated mitochondria (Evtodienko et al., 1996; Andre et al., 2000; Varbiro et al., 2001; Kidd et al., 2002), but the mitochondrial target of taxanes remains elusive at the molecular level. Carre et al. (2002) very recently discovered that tubulin is an inherent component of mitochondrial membranes with a still unknown function, thereby providing a potential ligand for taxanes in mitochondria. Herein, we report that paclitaxel binds to isolated mitochondria, and we found that this binding can be increased in condition of activation of the mitochondrial respiratory state. Bcl-2 participates in this binding, and its role as intracellular target for taxanes is supported by the fact that Bcl-2 down-regulation is observed in a panel of paclitaxel-resistant A2780 cells and that paclitaxel sensitivity is partially restored by Bcl-2 overexpression. Finally, we noticed Bcl-2 down-regulation in a small series of ovarian cancer patients resistant to paclitaxel containing chemotherapy.

Materials and Methods

Drugs. Paclitaxel and docetaxel were kindly provided by Indena S.p.A. (Milan, Italy), dissolved in DMSO (stock solution, 10 mM) and used within 7 days. The final DMSO concentration never exceeded 0.1% (v/v) in either control or treated samples.

Cell Cultures. A2780 and T47-D cells were purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK). Cells, propagated as monolayer culture in 75-cm^2 tissue culture flasks, were trypsinized twice weekly and plated at a density of 8×10^4 cells/ml. All cultures were incubated at 37°C in a high-humidity, 5% CO_2/95% atmosphere. Culture media were chosen according to the suggestions of ECACC. A2780 cell lines resistant to paclitaxel (A2780 T10, T15, T20, T30, and T50) were generated in our laboratory by exposing cells to stepwise increasing concentrations of paclitaxel alone. The number indicates the paclitaxel concentration in which cells are maintained to keep the drug-resistant phenotype. These cell lines exhibit classical multidrug-resistance (MDR) phenotype with P-glycoprotein (P-gp) overexpression. To generate cell lines with paclitaxel resistance without MDR, we firstly exposed A2780wt cells to increasing stepwise concentrations of cyclosporin A (Sigma, St. Louis, MO) up to a concentration of 4 μg/ml. Cyclosporin was added daily. Thereafter, these cells were exposed to increasing stepwise concentrations of paclitaxel up to a final concentration of 100 nM. This cell line was then cloned in limiting dilution and cell clones were selected for the negative MDR-phenotype by using rhodamine 123 and flow cytometry as described previously (Ferlini et al., 2001).

Growth Experiments. For growth experiments, 20,000 cells/well were plated in 96-well flat-bottomed plates (Cultureplates; PerkinElmer, Boston, MA). After 24 h, media were replaced; after one washing, fresh media containing the drugs were added. Three independent experiments were performed in quadruplicates. After 72 h of culture in the presence of the tested compounds, plates were harvested and the number of viable cells was estimated, using the ATPlite kit (PerkinElmer) and a Topcount automated luminometer (PerkinElmer). The kit was applied according to the manufacturer's suggestions. For each drug/cell line, a dose-response curve was plotted, and the IC_{50} values were then calculated by fitting the concentration-effect curve data obtained in the three experiments with the sigmoid-E_{max} model using nonlinear regression, weighted by the reciprocal of the square of the predicted effect, as described previously (Motulsky and Ransnas, 1987).

Mitochondrial Isolation. To isolate mitochondria, A2780 cells were collected by centrifugation and resuspended in a cold solution containing 0.3 M mannitol, 10 mM potassium HEPES, pH 7.4, 0.2 mM EDTA, pH 7.4, and 0.1% fatty-acid free bovine serum albumin (MT-1 EDTA). Cells were homogenized on ice with a glass and Teflon Potter homogenizer. The homogenate was centrifuged twice at 600*g* for 5 min to remove the nuclear fraction, and the mitochondrial fraction was sedimented from the supernatant at 12,000*g* for 10 min. Isolated mitochondria were then washed and resuspended in MT-1 medium without EDTA. To hyperpolarize mitochondria, MT-1 was supplemented with 0.5 mM potassium phosphate/4.2 mM potassium succinate, as described previously (Narita et al., 1998).

Fluorescent Probes and Flow Cytometry. The flow cytometry method to monitor $\Delta\Psi_m$ has been validated in our laboratory and was described previously (Ferlini et al., 1999). Briefly, $\Delta\Psi_m$ was measured using the sensitive cationic probe 5,5′6,6′-tetraethylbenzimidazolcarbocyanine iodide (JC-1), purchased from Molecular Probes (Eugene, OR). JC-1 staining was performed by exposing isolated mitochondria to 5 μg/ml JC-1 at room temperature for 15 min.

For each run, 15,000 events were acquired using Lysis II software on a FACScan flow cytometer (BD Biosciences, San Jose, CA) equipped with a collection filter for the green emission (530/30 nm) and orange emission (585/42 nm) for JC-1 monomers and JC-1 aggregates, respectively. All fluorescence signals were collected in log mode. Electronic color compensation was used to compensate for cross-over between fluorochromes. Light-scatter changes were acquired in linear mode. Data are expressed as two-dimensional contour plots reporting orange signal (aggregate form) versus green signal (monomer form).

Binding of [^{14}C]Paclitaxel to Mitochondrial Proteins and Bcl-2. In experiments with [^{14}C]paclitaxel (Sigma), isolated mitochondria were incubated with or without 1 μM [^{14}C]paclitaxel. After 120 min of incubation, mitochondria were washed twice with cold phosphate-buffered saline and lysed on ice for 30 min in lysis buffer [10 mM HEPES, pH 7.4, 150 mM NaCl, 1% (v/v) Triton X-100, 25 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 2 mM sodium vanadate]. Mitochondrial proteins (50 μg) were precipitated with 10% TCA. Precipitated proteins were pelleted for 20 min at 12,000*g*. Pellets were dissolved in 3 ml of scintillation liquid (Insta-gel Plus; PerkinElmer Life Sciences) by vortexing, and incubated for 20 min in agitation before counting. For immunoprecipitation, 150 μg of mitochondrial lysate was incubated, after preclearing for 1 h with Sepharose-conjugated protein A, at 4°C

overnight with either 0.5 μg of rabbit polyclonal anti Bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA) or goat anti-rabbit IgG (Bio-Rad, Hercules, CA), with constant shaking, at 4°C. After immunoprecipitation, protein A-Sepharose (Sigma) was added for 2 h at room temperature, and then centrifuged for 10 min at 1000*g*. Pellet was washed three times with lysis buffer before being dissolved in 3 ml of scintillation liquid in experiments with [^{14}C]paclitaxel. Immunoprecipitates were checked by Western blot analysis for Bcl-2 using anti Bcl-2 monoclonal antibody (clone 100; Santa Cruz Biotechnology). To assess proteins immunoprecipitated with anti Bcl-2, experiments of coimmunoprecipitation were also carried out using rabbit anti Bcl-2 and goat polyclonal anti–voltage-dependent anionic channel (VDAC) (Santa Cruz Biotechnology). In such experiments, stringency of the immunoprecipitation was increased by performing four washes in an hypertonic lysis buffer containing 500 mM NaCl.

Stable Transfection of Bcl-2. The breast cancer cell line T47-D with inherent drug resistance and the A2780TC1 and TC3 subclones with acquired drug resistance were transfected with pUSEamp(+)-Bcl-2 vector or with pUSEamp(+) (Upstate Biotechnology, Lake Placid, NY) using 2 μg of DNA per 1×10^6 cells. DNA was electroporated using a Gene Pulser (Bio-Rad) at 350 V, 500 μF. Cells were allowed to recover for 48 h, after which the media was removed and replaced with fresh media containing 1.5 mg/ml G418 (Sigma). Fresh G418-containing media was added every 3 days until visible colonies appeared. After three to four passages, cells were collected and Bcl-2 Western blot analysis was performed to evaluate at the same time Bcl-2 expression and paclitaxel sensitivity.

Western Blots and RT-PCR for Bcl-2 Expression. Cells were scraped into their culture media and pelleted. They were then washed twice with ice-cold phosphate-buffered saline, pH 7.4, and lysed on ice for 30 min using lysis buffer. After microcentrifugation for 15 min at 4°C, the supernatant was removed and protein concentration determined using the Bio-Rad protein assay. SDS-PAGE and Coomassie blue staining was performed for all samples as a control for sample integrity before Western blotting. Each protein sample (100 μg) was separated on a 12% SDS-polyacrylamide gel and electroblotted onto polyvinylidene difluoride membranes (Millipore Co., Bedford, MA). After electroblotting, the membranes were incubated in 6% nonfat dry milk in 1× Tris-buffered saline/Tween 20 (0.1 M Trizma base, 0.15 M NaCl, 0,05% Tween 20, pH 7.4) for blocking and then with the primary antibody in 3% nonfat dry milk in 1× Tris-buffered saline/Tween 20. The following primary antibodies were used: mouse monoclonal antibodies anti-Bcl-2 (clone 124, 1:200; DAKO, Glostrup, Denmark), anti-P-gp (clone C219, 1:200; Calbiochem, San Diego, CA), anti-survivin (clone D8, 1:200; Santa Cruz Biotechnology) and anti–β-actin (clone AC15, 1:3000; Sigma); and rabbit polyclonal antibodies anti–β-tubulin (1:500), anti-Bcl-x (1:500), anti-Bax (1:800; all from Santa Cruz Biotechnology). Secondary alkaline phosphatase and horseradish peroxidase-conjugated antibodies (1:2000) were purchased from Bio-Rad and visualization was performed with the 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium phosphatase substrate system (Kirkegaard and Perry Laboratories, Gaithersburg, MD) and the enhanced chemiluminescence-plus system (Amersham Biosciences, Buckinghamshire, UK), respectively. Enhanced chemiluminescence-plus was used for anti-Bcl-x and anti-survivin Western blots. Images of the blots were acquired using a HP ScanJet II scanner. Quantification was performed using Phoretix 1D Gel analysis software (Phoretix International, Newcastle upon Tyne, UK). Three biologically independent experiments were performed, and results were averaged after normalization for the loading control. To evaluate Bcl-2 mRNA expression level, we used a semiquantitative RT-PCR method validated in our laboratory and described previously (Marone et al., 1998).

Immunohistochemical Analysis of Bcl-2 Expression. The expression of Bcl-2 was immunohistochemically assessed in a series of 74 advanced ovarian cancer patients admitted in the Gynecologic Oncology unit of our Department. Median age was 53 years (range, 24–78 years). Forty-eight (65%) patients had stage III disease and 14 (19%) had stage IV disease. Most of the tumors were serous adenocarcinomas (59%) and showed a poor grade of differentiation (73%). All patients underwent platinum/paclitaxel-containing chemotherapy. Tumor tissue biopsies were obtained at first surgery, and chemotherapy followed surgery in all cases. Tissue specimens were fixed in formalin and paraffin-embedded according to standard procedures. Sections (4 μm) of representative blocks from each case were deparaffinized in xylene. For identification of Bcl-2 protein expression, the Envision-peroxidase system (DAKO) was used. Bcl-2 antigens were retrieved by microwave in 1 mM EDTA, pH 8.0, for 10 min. Clone 124 anti-human Bcl-2 primary antibody (1:20) in 1% bovine serum albumin/phosphate-buffered saline was used. Negative control for every experiment was done by replacing the primary antibody with albumin/phosphate-buffered saline. Positive control was represented by a section taken from a follicular lymphoma. For each specimen, five to seven sections were randomly selected. To quantify results of the immunoreactions, image analysis was performed, as described previously (Ferrandina et al., 2002). Briefly, the technical set-up included a Zeiss Axioskop (Zeiss, Jena, Germany) equipped with a Nikon Coolpix 950 digital camera (Nikon Corporation, Tokyo, Japan). Three 20× fields were chosen from each section so as to best reflect the overall immunostaining of the tumor contained on the entire slide. After acquisition with digital camera, the files were saved in TIF format, which allows compression (LZW algorithm) without discarding any data. The files were opened in Adobe Photoshop (v. 5.0; Adobe Systems, San Jose, CA) using a Macintosh 400-MHz G3 workstation (Apple, Cupertino, CA). The immunostained regions of interest were automatically selected and highlighted using the magic wand tool and an appropriate color tolerance level. The mean density value and the area (in pixels) of the immunostained regions were measured by the brightness filter tool, and a built-in calibration curve was constructed from the brightness filter readings and the known optical density values of calibrated wedges digitized with the same camera. The rest of the tumor tissue was subsequently selected using the inverse tool, and the relative area in pixels was calculated with the brightness filter tool and added to the immunostained area to obtain the total measured area. Then, the integrated density (ID) of the immunostaining was calculated as the product of the mean density value of the immunoreactive regions by the percentage of the immunostained tumor tissue. Image analysis was done without prior knowledge of the clinical and biologic parameters.

Results

To investigate possible direct effects of taxanes in isolated mitochondria, we monitored $\Delta\Psi_m$ in the presence of 1 μM docetaxel or paclitaxel using flow cytometry and the $\Delta\Psi_m$-sensitive dye JC-1. Mitochondria were kept either in the standard MT-1 medium or in MT-1 medium supplemented with 0.5 mM potassium phosphate and 4.2 mM potassium succinate (MT-2), to stimulate respiratory activity and hyperpolarize mitochondria (Narita et al., 1998). Results indicated that 1 μM docetaxel and paclitaxel (Fig. 1) did not alter $\Delta\Psi_m$ when mitochondria were kept in the MT-1 medium. When maintained in MT-2 medium, untreated mitochondria exhibited a progressive decrease in $\Delta\Psi_m$ to attain values similar to those kept in MT-1 after 150 min without relevant differences between the two taxanes. Taxane treatment in MT-2 medium induced an earlier loss of $\Delta\Psi_m$ in a manner that basal values (corresponding to those observed in MT-1 control mitochondria) were achieved after only 60 min.

To directly assess the mitochondrial binding of paclitaxel, we incubated isolated mitochondria in MT-1 and MT-2 media with ^{14}C-radiolabeled paclitaxel. After 2 h, mitochondria were washed and proteins were precipitated. In MT-1 medium, binding of paclitaxel yielded 18.6 ± 2.1 cpm/μg of

protein, whereas the binding increased nearly 9-fold after stimulation of the respiratory state, arriving at 163.2 ± 46.7 cpm/μg of protein (Fig. 2).

Because previous work suggested the possibility that paclitaxel binds to the disordered loop of Bcl-2, we looked at the binding of ^{14}C-radiolabeled paclitaxel to mitochondrial proteins (in MT-2 medium) immunoprecipitated with equivalent amounts of an anti-Bcl-2 and anti-rabbit IgG antibodies. The involvement of Bcl-2 in binding of paclitaxel was evidenced by the fact that proteins immunoprecipitated by the control anti-rabbit IgG antibody yielded only 1.93 ± 1.3 cpm/μg of protein, whereas those precipitated by the anti-Bcl-2 antibody gave 121.3 ± 19.8 cpm/μg of protein (Fig. 2).

To evaluate a possible role of Bcl-2 in taxane resistance, we established from A2780 cells a panel of cell lines resistant to increasing doses of either paclitaxel alone or paclitaxel/cyclosporin A. The addition of cyclosporin A served to prevent the appearance of resistant cells through the MDR phenotype (Sikic et al., 1997), because resistant cells obtained through exclusive exposure to paclitaxel always exhibited MDR phenotype with P-gp expression (Fig. 3). To better appreciate the profile of drug resistance, paclitaxel sensitivity of the used cell lines is reported in Fig. 3 as IC_{50} (drug concentration able to half-maximally reduce cell growth) values. As depicted in Fig. 4A, in whole cells, Bcl-2 is consistently down-regulated in all resistant cell lines with respect to parental paclitaxel-sensitive cells, regardless of the presence of the P-gp protein.

In terms of mRNA expression, cell lines obtained with continuous paclitaxel exposure at 10 and 30 nM (A2780 T10 and T30) exhibited a down-regulation in mRNA expression, whereas in A2780 T50 and A2780 TC clones, no changes at the level of Bcl-2 mRNA were detectable with respect to A2780wt cells (Fig. 4B).

To assess whether additional proteins regulating the apoptotic process could be involved in paclitaxel activity, we looked at the expression of Bax, Bcl-x, and the antiapoptotic protein survivin in the same cell lines (Fig. 4C). Bax and Bcl-x did not exhibit significant down-regulation with respect to A2780wt cells in paclitaxel-resistant cells. Only survivin was slightly up-regulated in resistant cells, but such up-regulation was not statistically significant (Fig. 4C).

Because cyclosporin promotes cancer progression and could induce drug-resistance independently from paclitaxel (Hojo et al., 1999), we also investigated Bcl-2 expression in an additional breast cancer cell line (T47-D) showing an inherent pattern of paclitaxel resistance. In addition, we found that in whole cells of this line, the protein was below the threshold of detectability by Western blot analysis (data not shown).

In A2780wt cells, A2780 TC1, and TC3 subclones and T47-D cells, equal amounts of mitochondrial proteins were immunoprecipitated with anti-Bcl-2. In keeping with results of whole cells, A2780wt expressed very high levels of Bcl-2 compared with paclitaxel-resistant cells (Fig. 5). On the other hand, a smaller Bcl-2 fragment near 24 kDa was detectable in all the three paclitaxel-resistant cell lines (Fig. 5). Concomitantly, we also immunoprecipitated equal amounts of mitochondrial proteins using anti-VDAC. If in A2780 cells Bcl-2 was intimately bound to VDAC, as demonstrated by coimmunoprecipitation, in paclitaxel-resistant cells, this binding was not detectable, because VDAC did not coimmunoprecipitate with Bcl-2 (Fig. 5). As control, in the same immunoprecipitates we assessed β-tubulin levels, recently identified as inherent component of the mitochondrial membranes interacting with VDAC (Carre et al., 2002). In this case, no differences were detectable between A2780wt cells and paclitaxel-resistant cell lines (Fig. 5), thereby indicating that in resistant cells, the interaction Bcl-2/VDAC was prevented, whereas the interaction β-tubulin/VDAC was unaffected.

To confirm the direct role of Bcl-2 in mediating taxane sensitivity, A2780 TC1 and TC3 subclones and T47-D cells were transfected with a pUSEamp(+)-Bcl-2 construct or pUSEamp(+) empty vector as control. Cells were then selected in media containing G418 and the obtained cell lines were examined for Bcl-2 expression (Fig. 6). All pUSEamp(+) vector-transformed cell lines expressed very low or undetectable levels of Bcl-2. In contrast, all pUSEamp(+)-Bcl-2 transfected cell lines displayed significant levels of Bcl-2 (Fig. 6). A statistically significant increase of paclitaxel sensitivity in all pUSEamp(+)–Bcl-2 transfected cell lines was observed. In fact, IC_{50} values obtained in such cell lines were significantly

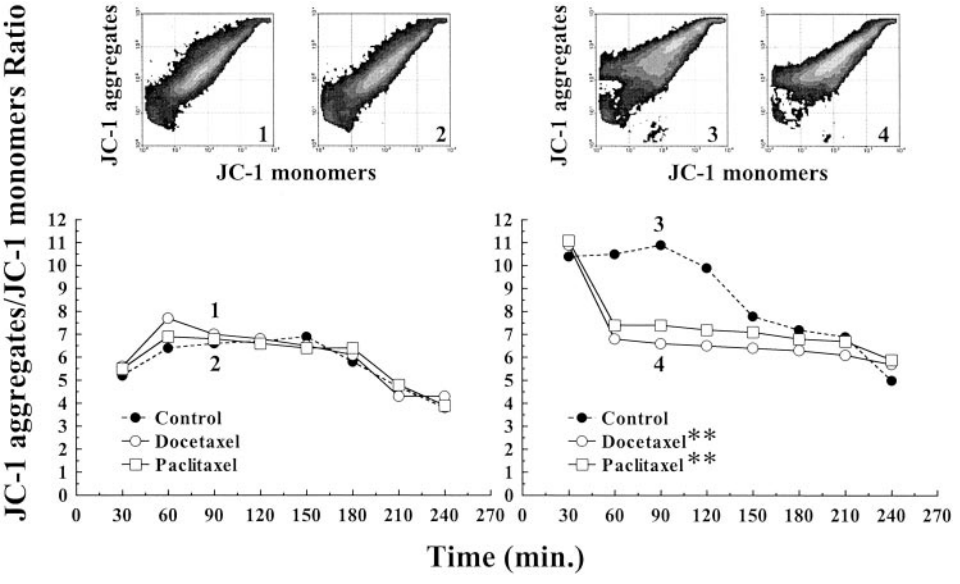

Fig. 1. Line charts representing the ratio between the mean channel of fluorescence produced by the aggregates of JC-1 (red fluorescence) and the monomers (green fluorescence) in control mitochondria (0.1% DMSO, ●) and mitochondria treated with 1 μM docetaxel (○) and 1 μM paclitaxel (□). Three independent experiments have been performed in triplicate but error bars have been omitted to add clarity to the graphs. **, $p < 0.01$; significant differences with respect to the control (ANOVA) for paclitaxel and docetaxel treatment in MT-2 at the time points 60, 90, and 120 min. In the insets, representative dot density plots (at time 90 min) of untreated mitochondria (1, 3) and treated (docetaxel; 2, 4) maintained in MT-1 (1, 2) and MT-2 (3, 4) medium, respectively.

lower than those concomitantly measured in the counterparts transformed with the empty vector (Fig. 6), thereby indicating that Bcl-2 overexpression is able to restore, at least in part, paclitaxel sensitivity.

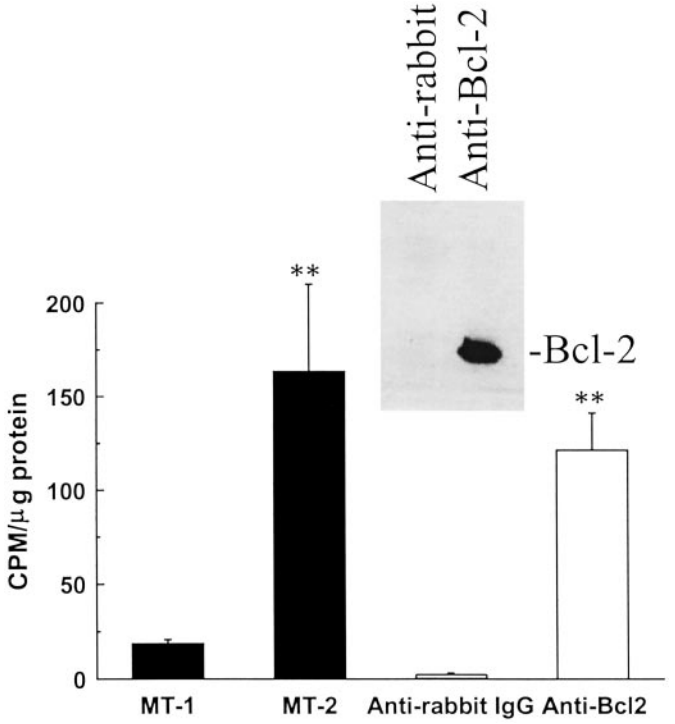

Fig. 2. Bar chart showing the [^{14}C]paclitaxel binding. Closed bars, isolated mitochondria kept in MT-1 or MT-2 media; open bars, obtained after immunoprecipitation of mitochondrial proteins from MT-2–treated mitochondria. Immunoprecipitation was performed either with a control antibody (anti-rabbit IgG) or the anti Bcl-2 antibody. Bar and error bars represent the mean and S.D. of triplicate samples. **, $p < 0.01$; the statistical significance of MT-2 versus MT-1 and anti-Bcl-2 versus control antibody (one-way ANOVA). In the inset, Western blot analysis for Bcl-2 to appreciate the efficiency of immunoprecipitation. However, [^{14}C]paclitaxel binding could be related to other proteins that coimmunoprecipitate with Bcl-2, such as VDAC or mitochondrial β-tubulin.

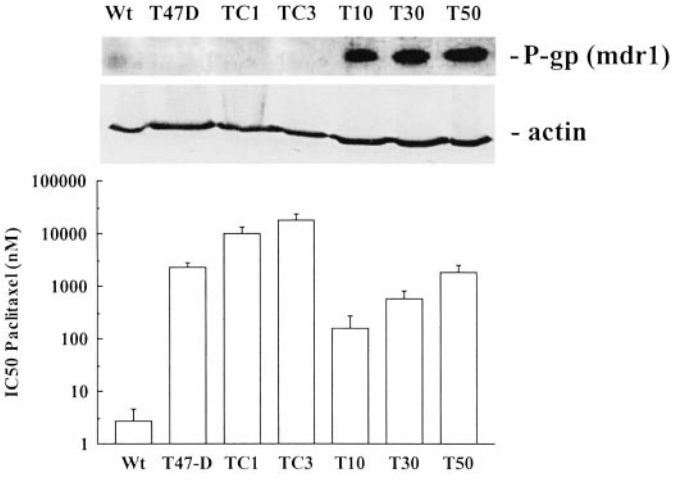

Fig. 3. Bar chart showing IC_{50} values obtained in the panel of cell lines used. IC_{50} values were obtained after 72 h of continuous exposure to paclitaxel. Bar and error bars are mean and S.D. of three independent experiments, respectively. In the insets, Western blot analysis for P-gp expression shows that A2780wt, T47-D, and A2780TC clones do not express detectable amount of P-gp, whereas cell lines obtained with single exposure to paclitaxel exhibit classic MDR phenotype with P-gp expression.

Finally, we looked at the Bcl-2 expression in a series of 74 ovarian cancer tissue specimens from patients administered a platinum/paclitaxel-containing regimen. Twenty of 74 (27%) cases were defined as resistant to platinum/paclitaxel chemotherapy. For each case, five to seven sections were immunostained with Bcl-2. Only two of 20 (10%) resistant patients exhibited Bcl-2 immunoreactivity, whereas the percentage rose to 22 of 54 (40.7%) in sensitive cases. Such difference resulted statistically significant ($\chi^2 = 6.3$; Fisher's exact test, $p = 0.0097$). Representative images, including the most positive section found in the paclitaxel-resistant group, are shown in Fig. 7. To quantify immunochemistry, image analysis was used to calculate the overall ID values. Results were 6.6 ± 7.8 and 34.7 ± 24.2 in resistant and sensitive patients, respectively. Difference between the two groups was statistically significant ($p = <0.006$ by ANOVA).

Discussion

Until now the antitumor activity of taxanes (at clinically relevant concentrations) has been assigned to the suppression of microtubule dynamics, leading to spindle paralysis and mitotic arrest (Jordan and Wilson, 1998) and all the relevant paclitaxel effects were assumed to result from microtubule-binding activity (Blagosklonny and Fojo, 1999). However, previous reports have suggested the possibility that taxanes could target isolated mitochondria in a microtubule-free context. In rat hepatocytes, paclitaxel reduces aerobic metabolism with a subsequent increase in ATP production via anaerobic glycolysis (Manzano et al., 1996). Because the same effect is present in isolated mitochondria, these authors have proposed that this drug directly affects the mitochondrial respiratory chain. Other authors have demonstrated that paclitaxel suppresses in mitochondria the closure of the megachannel called permeability transition (PT) pore (Evtodienko et al., 1996; Varbiro et al., 2001; Kidd et al., 2002). In keeping with these reports, we provide direct evidence that, along with microtubules, taxanes directly interact with mitochondria by inducing leakage of $\Delta\Psi_m$ in hyperpolarized mitochondria. It is noteworthy that when the mitochondrial respiratory state is energized (MT-2 medium), this occurs at taxane concentrations lower than those used in the aforementioned previous reports (range, 10–500 μM). The achievable serum concentration for paclitaxel at standard doses (135–175 mg/m^2) is in the range of 5 to 50 nM (Karlsson et al., 1999). Because taxanes are concentrated nearly 1000-fold within the cell (Andre et al., 2000), it is likely that an intracellular concentration of 1 μM can be clinically achieved and that paclitaxel can interact with mitochondria as well as with microtubules at the clinically used standard doses. Nevertheless, binding of paclitaxel seems enhanced in hyperpolarized mitochondria, possibly for conformational changes at the level of PT. This hypothesis has been supported by our findings, showing that MT-2 medium increases the binding of ^{14}C-radiolabeled paclitaxel to near 9-fold. Which is the mitochondrial target of taxanes? Screening a library of phage-displayed peptides to identify drug-binding sequence, Rodi et al. (1999) theorized that the disordered loop of Bcl-2 contains a binding sequence for paclitaxel. Using an anti-Bcl-2 antibody, we immunoprecipitated the vast majority of ^{14}C-radiolabeled paclitaxel bound to mitochondrial proteins, proving that Bcl-2 is an important com-

ponent of the mitochondrial target of taxanes. However, in such immunoprecipitates, we also detected VDAC and β-tubulin; therefore, we cannot conclude from our findings that paclitaxel directly binds to Bcl-2.

Bcl-2 acts physiologically as a proton efflux pump preventing conductivity of PT (Shimizu et al., 1998). As suggested previously (Voehringer, 1999), Bcl-2 binds to PT and acts as a "gatekeeper," thereby decreasing sensitivity to apoptotic stimuli. Our hypothesis is that taxanes bind to Bcl-2, thereby blocking its antiapoptotic function and allowing conductivity of PT. This view is in good keeping with previous reports showing that taxanes facilitate the opening of PT (Evtodienko et al., 1996; Kidd et al., 2002) and consequently the activation of apoptosis mediated by cytochrome *c* release (Andre et al., 2000).

A structural homology with bacterial colicins has been recognized for Bcl-2 family proteins (Schendel et al., 1997). Therefore, it is tempting to speculate that taxanes could originate from ancestral compounds probably developed by *Taxoidaceae* sp. progenitors for antimicrobic properties.

The relevance of Bcl-2 in mediating the cytotoxic effect of taxanes is supported by the fact that taxane-resistant cell lines display a consistent down-regulation of the protein. In cells resistant to high concentrations of paclitaxel, Bcl-2 down-regulation is dependent on posttranslational changes, possibly involved in Bcl-2 degradation. In support of this view, in Bcl-2 immunoprecipitates of isolated mitochondria from paclitaxel-resistant cells, we detected scanty levels of Bcl-2 compared with A2780wt cells along with a lower band of nearly 24 kDa. A similar band has been described previously as by-product of Bcl-2 generated by caspase-3 (Cheng et al., 1997). When immunoprecipitated with VDAC, in A2780wt, only the 27-kDa form of Bcl-2 was found, thereby indicating that only the whole protein is able to bind to VDAC. In paclitaxel-resistant cells, no detectable Bcl-2 coimmunoprecipitation with VDAC was noticed. On the other

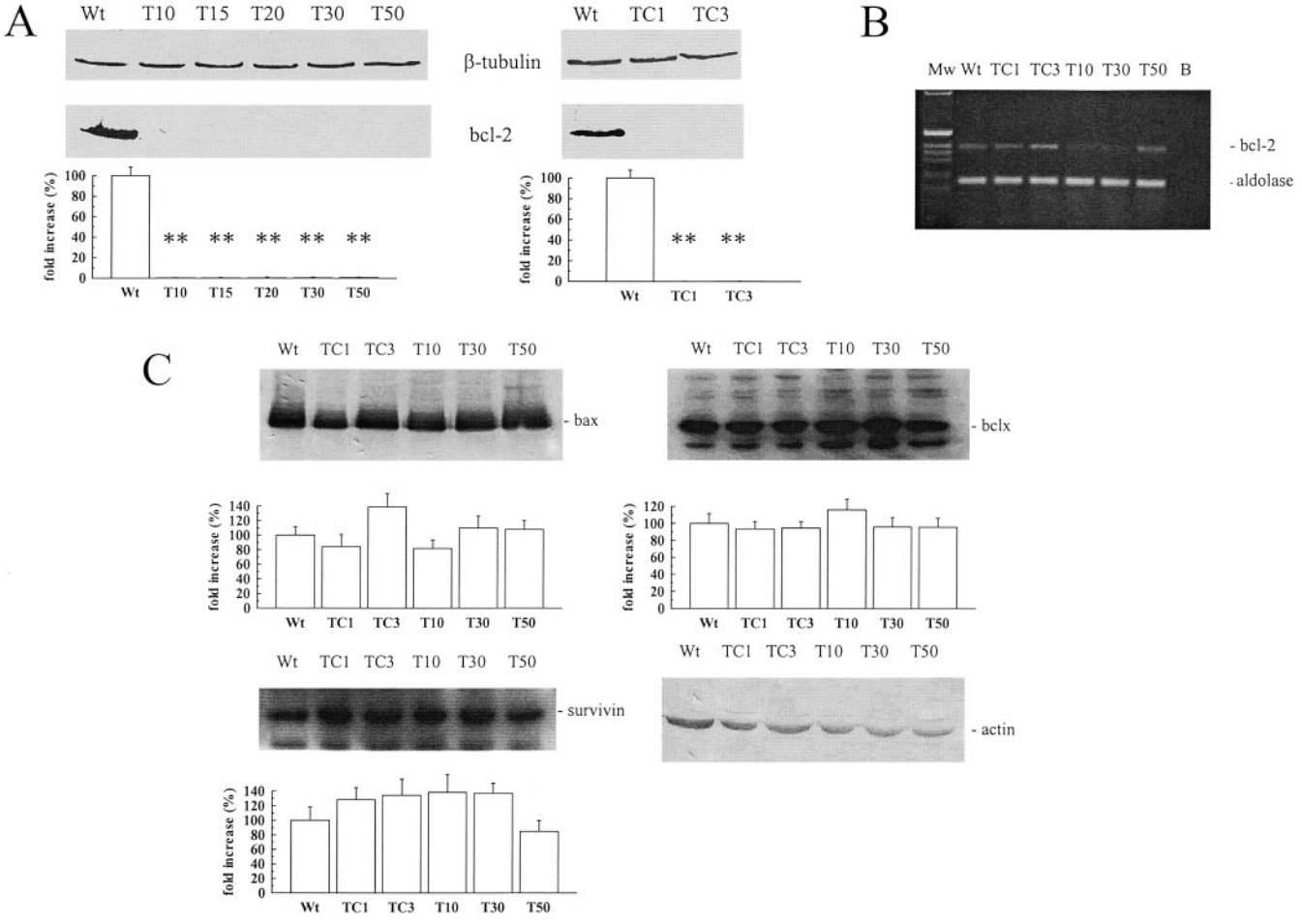

Fig. 4. A, Western blot analysis of Bcl-2 and β-tubulin expression in A2780wt cells and its paclitaxel-resistant counterparts. A2780 T10, T15, T20, T30, and T50 are MDR-positive cell lines resistant to 10, 15, 20, 30, and 50 nM of paclitaxel, respectively. A2780 TC1 and TC3 cells are MDR-negative cell clones resistant to 100 nM paclitaxel and 4 μg/ml cyclosporin A. Regardless of the MDR phenotype, all paclitaxel-resistant A2780 cells exhibit an impressive down-regulation of the Bcl-2 protein. Control β-tubulin expression was not affected by continuous drug exposure. Bar and error bars are mean and S.D. of three independent experiments. **, $p < 0.01$; statistically significant (ANOVA). B, semiquantitative RT-PCR for the mRNA of Bcl-2. Bcl-2 expression was quantified by normalizing the results for the housekeeping mRNA of aldolase. In A2780 T10 and T30 cells, a slight down-regulation at Bcl-2 transcriptional level was noticed, whereas in A2780 T50 cells and in the A2780 TC clones, there were no changes in terms of Bcl-2 mRNA. C, Western blot analysis of Bax, Bcl-x, survivin, and actin in A2780wt and its resistant A2780TC1, TC3, T10, T30, and T50 counterparts. β-Actin was added as an additional housekeeping control. At the bottom of each blot, in graphs, bar and error bars are mean and S.D. of three independent experiments. Statistical analysis revealed that all the measured changes do not attain the threshold of statistical significance ($p > 0.05$; ANOVA).

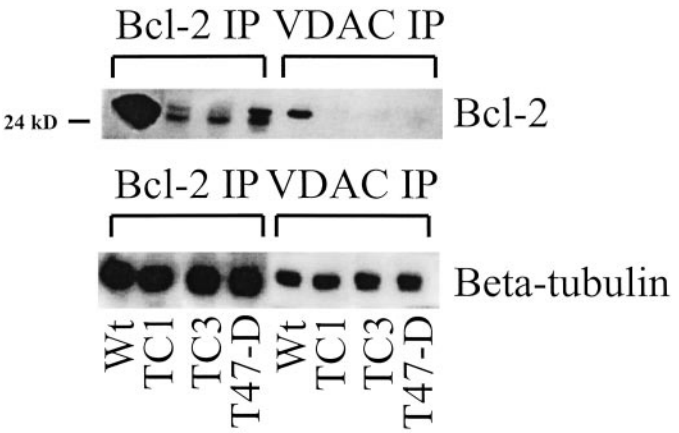

Fig. 5. Western blot analysis of Bcl-2 (top) and β-tubulin (bottom) in immunoprecipitates of mitochondrial proteins obtained from isolated mitochondria of A2780wt, A2780TC1, A2780TC3, and T47-D cells. In the left and right columns, polyclonal anti Bcl-2 and anti-VDAC antibodies have been used for the immunoprecipitation, respectively. In paclitaxel-resistant cells, a fragment of Bcl-2 is detectable with a size of nearly 24 kDa. In VDAC immunoprecipitates, no detectable evidence for Bcl-2 binding was noticed in drug-resistant cells. On the other hand, β-tubulin was equally detectable in Bcl-2 as well as in VDAC immunoprecipitates.

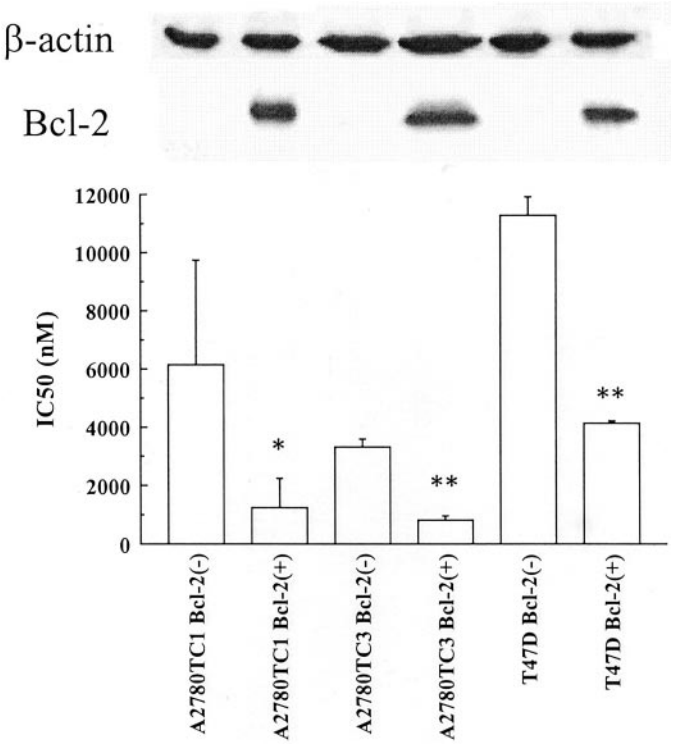

Fig. 6. Bar chart showing IC_{50} values in a panel of paclitaxel-resistant cell lines transformed with the pUSEamp(+)-empty vector or the pUSEamp(+)–Bcl-2 construct. Bar and error bars represent the mean and S.D. of the IC_{50} (concentration able to half-maximally reduce cell growth after 72 h of continuous drug exposure to paclitaxel) calculated in three independent experiments. *, $p < 0.05$; **, $p < 0.01$; statistically significant (pUSEamp(+)-empty vector versus pUSEamp(+)–Bcl-2 in each cell line, one-way ANOVA). Top, Western blot analysis for Bcl-2 expression in the same panel of cell lines.

hand, β-tubulin coimmunoprecipitates with Bcl-2 and VDAC in A2780wt as well as in paclitaxel-resistant cells, thereby suggesting no changes in the binding of β-tubulin to VDAC in paclitaxel-resistant cells. Tubulin has recently been revealed as an inherent mitochondrial membrane protein with an as-yet-unknown function (Carre et al., 2002). The presence of tubulin provides a "natural" target for taxanes. However, the absence of alteration at the level of VDAC/β-tubulin interaction in paclitaxel-resistant cells could lead one to predict that Bcl-2 acts as the actual modulator of mitochondrial sensitivity to paclitaxel. This direct role in mediating drug-resistance is strengthened by the fact that Bcl-2 overexpression was able to restore paclitaxel-sensitivity in paclitaxel-resistant cells.

Accordingly, when analyzing Bcl-2 immunoreactivity in our clinical setting, we found a low percentage of Bcl-2 positivity as well as low Bcl-2 expression in patients resistant to paclitaxel-containing chemotherapy. Several groups of researchers have reported that Bcl-2 is positively correlated with prognosis of ovarian cancer (Henriksen et al., 1995; Diebold et al., 1996; Herod et al., 1996; Chan et al., 2000). Unfortunately, authors in those reports were unable to describe the relationship between Bcl-2 status and response to chemotherapy. Although it has been proposed that Bcl-2 overexpression can prevent chemotherapy-induced apoptosis and contribute to drug resistance, the association between Bcl-2 and drug susceptibility in ovarian cancer is still controversial (Baekelandt et al., 1999; Kassim et al., 1999; Mano et al., 1999; Schuyer et al., 2001). In particular, the use of different drug combinations, with or without the inclusion of paclitaxel, could aid in absolving the controversies. For instance, two studies have reported that in ovarian cancer, an inverse correlation between Bcl-2 expression and response to chemotherapy exists (Kassim et al., 1999; Mano et al., 1999). This inverse correlation seems to be in contrast to our findings, but the discrepancy is only apparent, because the patients received chemotherapy based on cisplatin and excluding taxanes. This also highlights the fact that mechanisms of drug resistance can be diverse and dependent on the chemo-

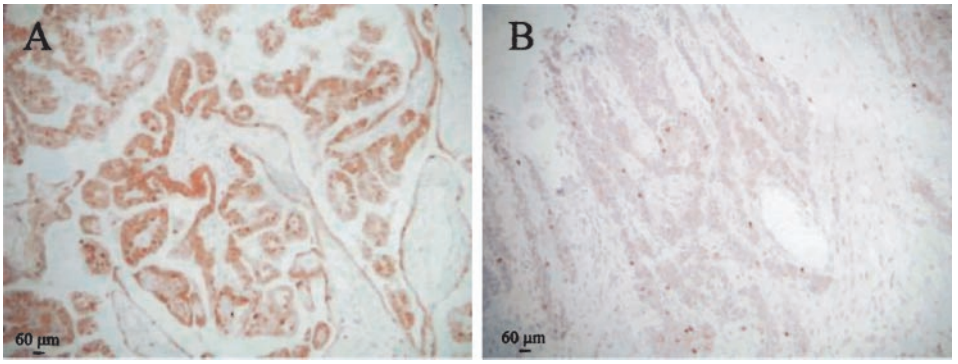

Fig. 7. Photomicrographs of sections taken from human ovary adenocarcinoma (125× magnification). A and B are low-grade cancers coming from patients sensitive or resistant to paclitaxel, respectively. Although staining is present in >75% of cells in both cases, Bcl-2–immunoreactive products are strikingly more intense in A than in B. Remarkably, B was the specimen with the highest Bcl-2 immunoreactivity in the paclitaxel-resistant setting. Counterstained with Mayer's hematoxylin. Scale bar, 60 μm.

therapy composition. Therefore, the role of Bcl-2 as predictor of chemotherapy response should be re-examined in the light of our current finding and according to the specific drug composition of chemotherapy regimens.

Because until now Bcl-2 down-regulation has been regarded as a factor of chemosensitivity linked to enhancement of drug-induced apoptosis, an antisense Bcl-2 (Genasense) is currently undergoing phase I/II clinical trials also in combination with taxanes (Chi et al., 2000; Tolcher, 2001). Our findings warn that Bcl-2 down-regulation could be harmful in patients treated with taxanes.

In summary, we have demonstrated that taxanes interact with Bcl-2 in mitochondria at clinically achievable doses, and Bcl-2 down-regulation seems to be associated, at least in ovarian cancer, with taxane-resistance. Clinical studies are now needed to ascertain whether Bcl-2 expression can be regarded as a potential factor of chemosensitivity for taxanes, and it is essential to correlate Bcl-2 expression and taxanes-sensitivity in other solid malignancies. Finally, the insight into taxane/Bcl-2 interaction could contribute to the development of a new generation of taxanes able to interact with Bcl-2 in a more efficient manner.

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