

## Chemotherapy drug response in ovarian cancer cells strictly depends on a cathepsin D–Bax activation loop

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### Abstract

The ovarian cancer cell lines A2780 (wild-type p53) and NIHOVCAR3 (mutated p53) showed, respectively, sensitivity and resistance towards several chemotherapy drugs. We hypothesized that the two cell lines differ in their ability to activate the intrinsic death pathway and have, therefore, dissected the lysosome–mitochondrion signalling pathway by pharmacological inhibition or genetic manipulation of key regulators and executioners. Biochemical and morphological confocal fluorescence studies showed that: (1) In A2780 cells bcl-2 is expressed at an undetectable level, whereas Bax is expressed at a rather high level; by contrast, bcl-2 is highly expressed and Bax is expressed at extremely low levels in NIHOVCAR3 cells; (2) Chemotherapy treatment reduced the expression of bcl-2 in NIHOVCAR3 cells, yet these cells resisted to drug toxicity; (3) Cathepsin D (CD), not cathepsin B or L, mediates the activation of the mitochondrial intrinsic death pathway in A2780 cells; (4) Lysosome leakage and cytosolic relocation of CD occurs in the chemosensitive A2780 cells, not in the chemoresistant NIHOVCAR3 cells; (5) Bax is essential for the permeabilization of both lysosomes and mitochondria in A2780 cells exposed to chemotherapy drugs; (6) CD activity is mandatory for the oligomerization of Bax on both mitochondrial and lysosomal membranes; (7) Bax activation did not occur in the resistant NIHOVCAR3 cells despite their high content in CD. The present data are consistent with a model in which on treatment with a cytotoxic drug the activation of a CD–Bax loop leads to the generalized permeabilization of lysosomes and eventually of mitochondria, thus reaching the point of no return, and culminates with the activation of the caspase cascade. Our data also imply that dysfunctional permeabilization of lysosomes contributes to the development of chemoresistance.

**Keywords:** chemoresistance • bcl-2 • apoptosis • lysosomes • mitochondria • VP16 • taxol • lysosome permeabilization

### Introduction

Ovarian cancer is the leading cause of death among patients bearing gynaecological cancers [1]. Treatments of epithelial ovarian carcinomas include surgery and chemotherapy. Unfortunately, after showing initial benefit out of the treatment, most patients succumb as a consequence of cancer relapse and dissemination. Survival rates of patients bearing advanced ovarian cancer are generally low (<30% at 5 years) because of the rapid development of resistance to chemotherapy drugs [2–4]. Recurrent disease arises from emerging clones in which chemotherapy drugs

fail to induce cell death because of overexpression of multidrug effluxing pumps, hyper-expression of the anti-apoptotic protein bcl-2, mutations of the oncosuppressor p53 [5–8] or other unknown factors. Recently, it has been shown that resistance to *cis*-platinum, the drug of first choice in the chemotherapy of ovarian cancers, was associated with its sequestration within lysosomes and subsequent active extrusion *via* exocytosis [9]. Most chemotherapy drugs act *via* activation of the intrinsic pathway of apoptosis, which is initiated by a caspase-activating multimolecular complex (the apoptosome) contributed by pro-apoptotic factors released from mitochondria [10, 11]. Defective formation of the apoptosome has been linked with the chemoresistance phenotype of ovarian cancer cells [12]. Mitochondrial permeabilization is induced under cytotoxic conditions and is mediated by proteins of the Bcl-2 family, such as bid and Bax. The latter proteins are proteolytically activated by caspase 8 [13, 14] and also by lysosomal cathepsins B, L and D (CB, CL, CD) [15–17]. CB, CL and CD normally

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reside in endosomes and lysosomes, where they accomplish the extensive or limited proteolysis of substrates at acid pH, thus contributing to cell protein turnover and homeostasis and activation or degradation of biologically active molecules [18]. Recently, these lysosomal cathepsins have been shown to actively participate in the cell death program following their translocation from lysosome into the cytosol [19]. For instance, CB has been shown to mediate caspase-independent cell death by microtubule stabilizing agent in lung cancer cells [20] and CD has been shown to mediate the caspase-dependent death pathway triggered by various DNA-damaging agents including etoposide [21, 22]. These findings underscore the importance of a lysosome-mitochondrion axis in the control of the death signal triggered by cytotoxic stress [23, 24]. Therefore, the function of these two organelles should also be considered when assessing the sensitivity or resistance towards apoptosis-inducing chemotherapy drugs in cancer cells. In this respect, it is to note that resistance to *cis*-platinum in ovarian cancer cells was found associated with abnormalities of the lysosomal apparatus [9]. The present study was aimed at elucidating the potential role and the mechanism of action of CB, CL and CD in the cytotoxic response to etoposide (VePesid, VP16, Sigma-Aldrich Corp., St Louis, MO, USA) and Paclitaxel (Taxol, Bristol-Myers Squibb, New York, NY, USA), two drugs that have a well-documented clinical efficacy against ovarian cancers that manifest resistance to platinum-based therapy [4, 25]. We analysed the cytotoxic pathways of these drugs in two human ovarian cancer cell lines, namely A2780 and NIHOVCR3 (OVCAR), that have been reported to express normal or mutated p53, respectively [26, 27]. Both VP16 and taxol turned out to be toxic to A2780, not to OVCAR cells. We found that chemosensitivity is associated with Bax-mediated permeabilization of lysosomes and mitochondria and that CD activity is mandatory for Bax activation and translocation onto lysosomes and mitochondria. Our data also indicate that Bax deficiency may result in the inability to permeabilize the lysosomes under toxic conditions, thus representing a primary defect that confers resistance towards chemotherapy drugs in cancer cells. We conclude that the CD-Bax cooperation is a critical determinant of chemosensitivity or chemoresistance in cancer cells.

## Materials and methods

Chemicals were of analytical grade. Unless otherwise specified all reagents, including antibodies, were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA).

### Cell culture and treatments

The human ovarian cancer cell lines A2780 and NIHOVCR3 (OVCAR) were obtained from the American Type Culture Collection (Rockville, MD, USA) and grown under standard culture conditions (37°C; 95% air; 5% CO<sub>2</sub> ratio) in RPMI 1640 medium supplemented with 10% heat-inactivated foetal bovine serum, 2 mM glutamine and antibiotics. For the experiments,

cells were seeded at  $30 \times 10^3/\text{cm}^2$  in Petri dishes and let adhere for 24 hrs prior to any treatment. Paclitaxel (Taxol) or etoposide (VP16) was added in fresh medium at concentrations ranging from 10 to 100 nM or 5 to 25  $\mu\text{M}$ , respectively. Treatments also included 100  $\mu\text{M}$  pepstatin A (Pst; added 16 hrs in advance) or 10  $\mu\text{M}$  CA074Me (Bachem AG, Bubendorf, Switzerland; added 1 hr in advance) or 10  $\mu\text{M}$  E-64d (Bachem AG; added 1 hr in advance) as indicated. Preliminary experiments were performed with different concentrations of the cathepsin inhibitor (Pst, 50–200  $\mu\text{M}$ ; 5–20  $\mu\text{M}$  CA074Me; 10–50  $\mu\text{M}$  E-64d) and the optimal concentration was chosen based on the most effective enzyme inhibition and the minimal cytotoxic side effects, also in accord with literature data. Pst was diluted from a stock solution in dimethylsulfoxide (working concentration of the solvent did not exceed 0.1% v/v, a concentration that was not toxic to cells).

### Experimental evidence of apoptosis and necrosis

At the time indicated, adherent cells from control and treated cultures were harvested and counted in triplicate. Necrotic cells were detected by trypan-blue staining. Apoptosis-associated chromatin alterations were detected by staining the cells with the DNA-labelling fluorescent dye 4-6-diamidino 2-phenylindol-dihydrochloride (DAPI, 1:500 in phosphate buffered saline (PBS)/0.1% Triton X-100, 4% foetal bovine serum). Alternatively, apoptotic cells were revealed by *in situ* terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling (TUNEL) assay performed with the 'In situ Cell Death Detection' fluorescent kit (Roche Diagnostics Corporation Indianapolis, IN, USA) following manufacturer's instructions. Cell death was estimated by cytofluorometric quantification of annexin V positivity. In brief, cells were collected, washed in PBS and incubated for 15 min. at room temperature with 2  $\mu\text{l}$  annexin V-FITC (Alexis Laboratories, San Diego, CA, USA) and 93  $\mu\text{l}$  of buffer (10 mM HEPES/NaOH pH 7.4, 140 mM NaCl, 2.5 mM CaCl<sub>2</sub>). FITC Fluorescence on cell surface was measured with a FacScan flow cytometer (Becton Dickinson, Mountain View, CA, USA) equipped with a 488-nm argon laser. At least 10,000 events were analysed for each sample. Data were interpreted with the winMDI software.

### Small interference RNA (siRNA) gene silencing

CD and Bax gene expression was post-transcriptionally silenced using the siRNA technology. Duplexes of 27-nucleotide siRNA without overhang dTdT (deoxythymidine) were synthesized by MWG Biotech AG (Washington, DC, USA). The sense strand of a siRNA was designed corresponding to the positions 724–742 relative to the start codon of the CD mRNA, as previously reported [28, 29]. The same technology was employed for post-transcriptional silencing of Bax using the sequence 5'-GGAACUGAUCAGAACCAUCAUGGGCUG-3' for the sense strand of siRNA [29]. An inefficient control-duplex oligonucleotide was used as a negative control of transfection (sham). Transfection was performed with Lipofectamine 2000 (Invitrogen Corp., Carlsbad, CA, USA) following the manufacturer's protocol. Cells were then incubated for 24 hrs in fresh medium prior to start of the incubation with VP16. Effective down-regulation of protein synthesis was ascertained by immunoblotting.

### Cathepsin D activity

To assay CD activity an aliquot of cell homogenate was incubated for 12 hrs at 37°C in a formate-acetate buffer (12.5 mM, pH 3.65) with 1  $\mu\text{g}$  aminomethylcoumarine-haemoglobin substrate in 50  $\mu\text{l}$  final volume [30].

The assay was run on a multiwell plate and fluorescence was read in a spectrofluorometer at 365 and 460 nm excitation and emission wavelengths, respectively. To prove that substrate was hydrolysed by CD, the specific inhibitor Pst was added to parallel samples.

## Fluorescence microscope imaging

Images were captured with a Zeiss (Arese, Italy) fluorescence microscope equipped with a digital camera or with Leica DMIRE2 confocal fluorescence microscope (Leica Microsystems AG, Wetzlar, Germany) equipped with Leica Confocal Software v. 2.61. Coverslips with adherent cells stained as detailed below were mounted in mowiol (1% in PBS). For each experimental condition three coverslips were prepared. At least four fields in each coverslip were examined by two independent investigators. Representative images of selected fields are shown. Data were reproduced in at least three separate experiments. Immunofluorescence staining was performed as previously reported using as primary antibodies a rabbit polyclonal anti-human CD [30], a monoclonal antibody against human LAMP1 (BD Bioscience, CA, USA) and a specific rabbit polyclonal anti-Bax antiserum (Cell Signaling Technology, Denver, MA, USA). Immunocomplexes were revealed by appropriate FITC- or TRITC-conjugated goat-anti-rabbit or goat-antimouse IgG secondary antibodies.

## Lysosomes and mitochondria permeabilization studies

Lysosomal membrane integrity was tested by using the lysosomotropic fluorochrome acridine orange (AO). Cells on coverslips were loaded with AO (15 min. incubation, final concentration 15 (g/ml), exposed to VP16 for up to 24 hrs, and immediately observed and photographed under the fluorescence microscope. Mitochondrial membrane integrity was tested by using mitotracker red (Invitrogen Corp.). In this case, cells on coverslips were incubated with 0.2 (l/ml of mitotracker solution for 15 min. at 37°C, fixed in 3.7% paraformaldehyde for 30 min., permeabilized with 0.2% Triton X-100 for 20 min. and further processed for DAPI or immunofluorescence staining.

## Immunoblotting

Cells were homogenized in buffer containing detergents and protease inhibitors and an aliquot corresponding to 50 µg of proteins was denatured with Laemmli sample buffer, separated by electrophoresis on a 12.5% polyacrylamide gel and then electroblotted onto nitrocellulose membrane (Biorad, Hercules, CA, USA). The filter was probed with specific monoclonal antibodies against CD (monoclonal from EMD Biosciences, Calbiochem, San Diego, CA, USA), or mouse monoclonal antibody bcl-2 (Santa Cruz Technology, Inc., Santa Cruz, CA, USA) or rabbit polyclonal anti-human Bax antiserum (Cell Signaling Technology, MA, USA) or mouse monoclonal anti-human p53 (DO-1 Santa Cruz Biotechnology, Inc.) or mouse monoclonal α-actin (Sigma-Aldrich Corp.). Immunocomplexes were revealed by incubation with peroxidase-conjugated goat-antimouse or goat-anti-rabbit, as appropriate, antibody and subsequent peroxidase-induced chemiluminescence reaction (Biorad) [30]. Intensity of the bands was estimated by densitometry analysis (Quantity one software). The densitometry ratio between the relevant protein and actin (for loading normal-

ization) is assumed as a measure of protein expression. Western blotting data were reproduced in at least three independent experiments.

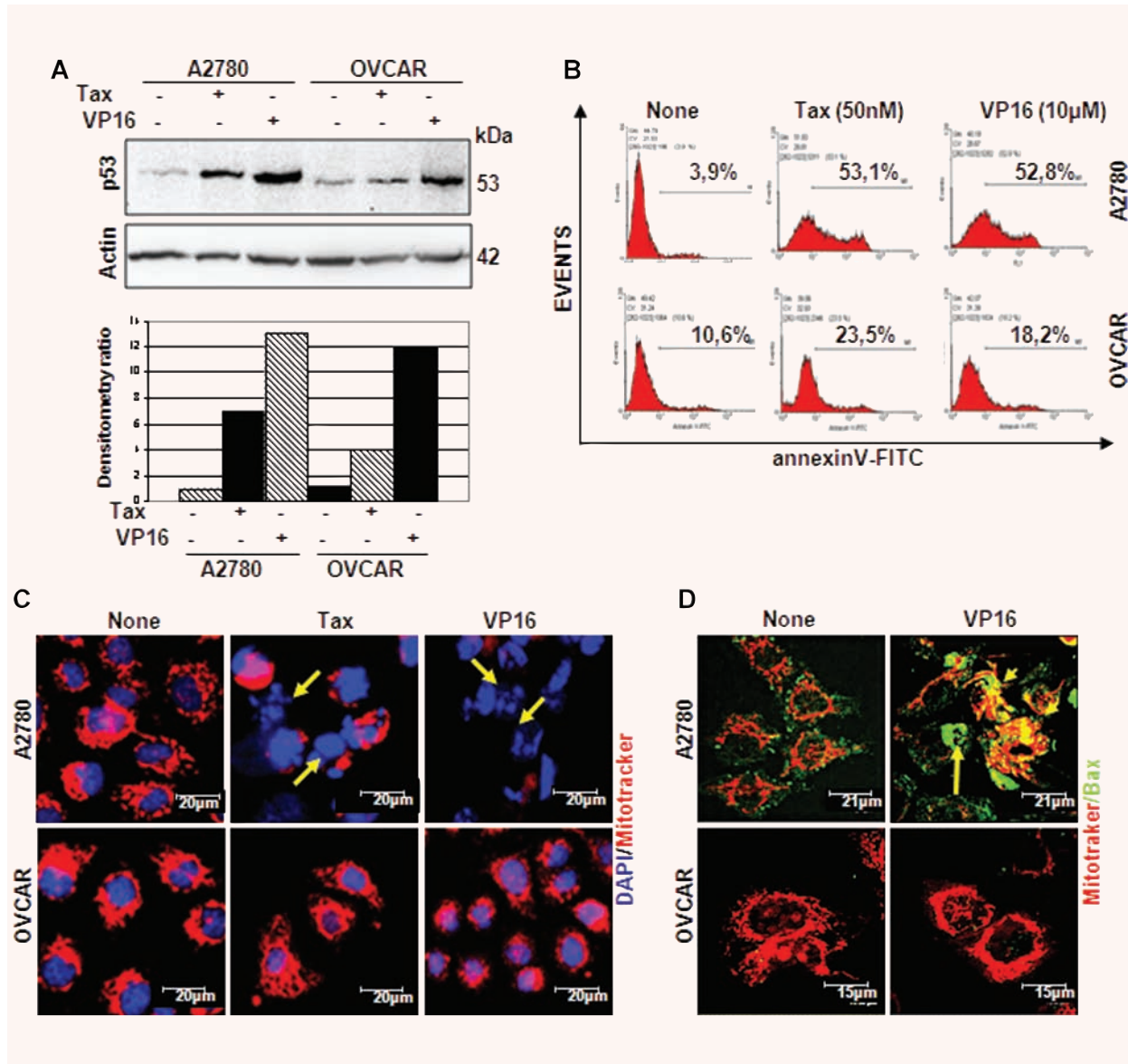
## Statistical analysis

All experiments were independently replicated at least three times. Data are presented as means ± S.D. The Microsoft Excel XLStats software was used. Significance was taken at a *P*-value less than 0.05.

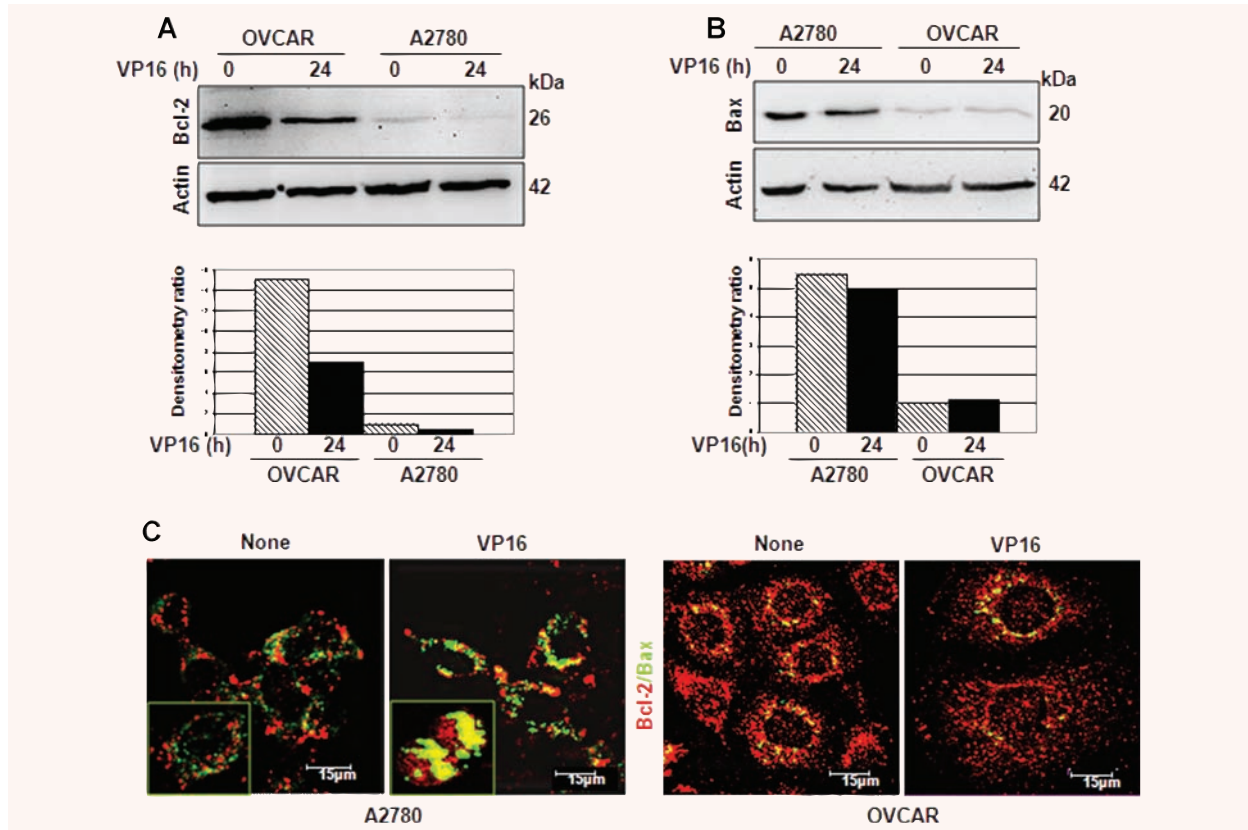
## Results

### Chemotherapy drugs activate a Bax-mediated intrinsic death pathway in A2780 cells not in OVCAR cells

Preliminary experiments in which the cells were exposed for 24 to 72 hrs to increasing doses of *cis*-platinum (0.5–5.0 µM), resveratrol (10–200 µM), VP16 (1–20 µM) or taxol (10–100 nM) revealed that A2780 cells are sensitive whereas OVCAR cells are resistant towards all tested chemotherapy drugs. These drugs exert their cytotoxicity through the activation of the intrinsic apoptosis pathway [12, 22, 28, 31, 32]. To identify the molecular basis for the different sensitivity to chemotherapy drugs, we first explored the functionality of this pathway in the two cell lines. VP16, a drug widely used in the therapy of ovarian cancers that show resistance to *cis*-Platinum [25], was employed as a paradigm of anticancer drug. In some experiments we also included taxol, which also is employed in the second line therapy of drug resistant ovarian cancers [4]. For the cytotoxic treatments, we chose the lowest dose of VP16 and of taxol that revealed to be most effective in a 24-hr incubation, *i.e.* 10 µM VP16 and 50 nM taxol, in the chemosensitive A2780 cell line. Both the treatments elicited an increased expression of p53 in the two cell lines, although this effect was prominent in A2780 cells (Fig. 1A). Counting of viable cells (data not shown and Fig. 3) and flow cytometry quantification of cell surface phosphatidylserine (which mirrors apoptosis) indicated that after such a treatment a large proportion (~50%) of A2780 cells underwent apoptotic cell death, whereas the cytotoxic effect of both drugs was negligible in OVCAR cells (Fig. 1B). In a separate experiment, the co-treatment of A2780 cells with the antiproliferative drugs and the pan-caspase inhibitor ZVAD-fmk resulted in absence of cytotoxicity, proving that cell death occurred *via* true apoptosis (not shown; see below). Parallel cultures in coverslips were double-stained with DAPI and mitotracker red, which allowed assessing the occurrence of mitochondrial membrane permeabilization in apoptotic cells. The latter were identified on the basis of the typical chromatin alterations (*i.e.* condensation and fragmentation) demonstrated by the fluorescent dye DAPI. The images in Fig. 1B show nuclei with condensation and fragmentation of the chromatin (arrows) in treated A2780, not in OVCAR cells. Of note, apoptosis in A2780 cells was



**Fig. 1** Chemotherapy drugs activate the intrinsic death pathway in A2780 cells, not in OVCAR cells. NIHOVCAR3 (OVCAR) and A2780 ovarian cancer cells were treated or not for 24 hrs with 10 µM VP16 or 50 nM taxol (Tax) and p53 expression and cytotoxicity together with mitochondria integrity were evaluated. The experiments were repeated four times in triplicate. Representative data are shown. **(A)** Western blotting analysis (and relative densitometry) of p53 expression. Both drugs effectively up-regulated the expression of p53 in both the cell lines. **(B)** Outer membrane exposition of phosphatidylserine, an indicator of apoptosis, was measured by cytofluorometry in control and chemotherapy drug-treated cells labelled with annexin V-FITC. Cytofluorograms demonstrate occurrence of cell death in treated A2780 cells. **(C)** Cells adherent on coverslips were labelled with mitotracker red, then fixed and labelled with DAPI. Images demonstrate occurrence of mitochondrial permeabilization in apoptotic cells (arrows) in treated A2780 cultures. **(D)** Cells adherent on coverslips and exposed for 24 hrs to VP16 were stained with mitotracker red, then fixed and further processed for Bax immunofluorescence. In untreated cells mitotracker staining demonstrates the integrity of mitochondria, whereas Bax staining is faintly detected and diffused as expected for inactive Bax. On treatment with VP16, in OVCAR cells no changes in mitotracker red and Bax staining is evident, whereas in A2780 cells it is clearly evident a strong and definite staining of Bax along with loss of mitotracker staining. In the latter case, the oligomerization of Bax on mitochondrial membrane, as shown by merged fluorescence with the mitotracker dye (arrow), can be appreciated in some cells.



**Fig. 2** Effect of VP16 on the expression and sub-cellular localization of Bax and bcl-2 in ovarian cancer cells. **(A)** The Western blotting shows that bcl-2 is highly expressed in OVCAR cells and very barely expressed in A2780 cells. VP16 greatly reduces (~50 %) the cellular content of bcl-2 in OVCAR cells, whereas it does not affect the expression of this protein in A2780 cells (see densitometry). **(B)** The Western blotting shows that Bax is highly expressed in A2780 and faintly detectable in OVCAR. Bax expression is not affected by VP16 (see densitometry). **(C)** Immunolocalization of bcl-2 and Bax. Both proteins appear as barely detectable spots diffused in the cytoplasm in control cells. On treatment with VP16 only in A2780 cells Bax appears as strongly intense spots in confined region of the cytoplasm, indicative of conformational activation and granular-like aggregation of the molecules. Bcl-2 does not co-localize with such Bax-positive aggregates.

associated with mitochondrial permeabilization, as demonstrated by loss of mitotracker staining (Fig. 1C). We then tested whether Bax, a bcl-2 family member involved in mitochondrial permeabilization, was differently targeted upon chemotherapy drug treatment in the two cell lines. The images shown in Fig. 1D clearly indicate that in A2780, not in OVCAR, Bax localizes onto mitochondrial membranes (and this coincides with permeabilization of mitochondria, as demonstrated by loss of mitotracker staining) in response to VP16.

### Bcl-2 and Bax are differently modulated in A2780 and OVCAR cells in response to cytotoxic treatments

Bcl-2 and Bax are members of the same family with opposite regulatory function in apoptosis, being bcl-2 anti-apoptotic and Bax pro-

apoptotic [33]. The coordinate expression of these two proteins is considered a determinant factor in chemosensitivity and chemoresistance of ovarian cancer cells [34–39]. Western blotting assessment of the relative expression of bcl-2 and Bax in response to the cytotoxic drug VP16 shows that: (1) bcl-2 is highly expressed in OVCAR, and this expression is strongly reduced in cells exposed for 24 hrs to VP16, whereas A2780 basally express this protein at very low level (Fig. 2A); (2) Bax, on the other hand, is expressed at fairly high level in A2780 cells whereas it is faintly detectable in OVCAR cells (Fig. 2B) and (3) both in A2780 and OVCAR cells the level of Bax expression does not change significantly after a 24-hrs exposure to VP16 (Fig. 2B). The changes in the cellular level of p53 and bcl-2 (Figs 1A and 2A and B) indicate that chemotherapy drugs reached in both cell types a concentration sufficient to alter the metabolism and genetic expression, yet only A2780 showed responsiveness. It is of note that OVCAR cells resisted to the cytotoxic treatments despite the marked down-regulation of bcl-2. To better assess the

role of bcl-2 in chemoresistance, VP16 was administered to OVCAR cells in which the synthesis of this protein had been genetically silenced by specific siRNA transfection. This experiment demonstrated that down-regulation of bcl-2 expression is not sufficient to sensitize OVCAR cells towards the chemotherapy drug (not shown). Bax exerts its pro-apoptotic function by relocating from the cytosol, where it is retained in its inactive conformation, onto the mitochondrial membranes where it oligomerizes and produces transient pores [40]. Bcl-2 exerts its anti-apoptotic function by hetero-dimerization with Bax, thus preventing its oligomerization on mitochondria [41]. We looked at the localization of these proteins in A2780 and OVCAR cells exposed to VP16. The images in Fig. 2C show that in both control A2780 and OVCAR cells bcl-2 and Bax proteins are distributed throughout the cytoplasm and, apparently, do not form complexes. It is to note that Bax is revealed by the antibody only upon a conformational activation that exposes its N-terminus [15]. In A2780 cells, but not in OVCAR cells, the treatment with VP16 leads to the conformational activation of Bax that is revealed as macro-aggregates intensely positive (Fig. 2C). Apparently, bcl-2 does not co-localize within this macro-aggregates. Altogether, the present data indicate that expression and conformational activation of Bax, rather than bcl-2 expression, are the determinant factors in the response to chemotherapy drugs.

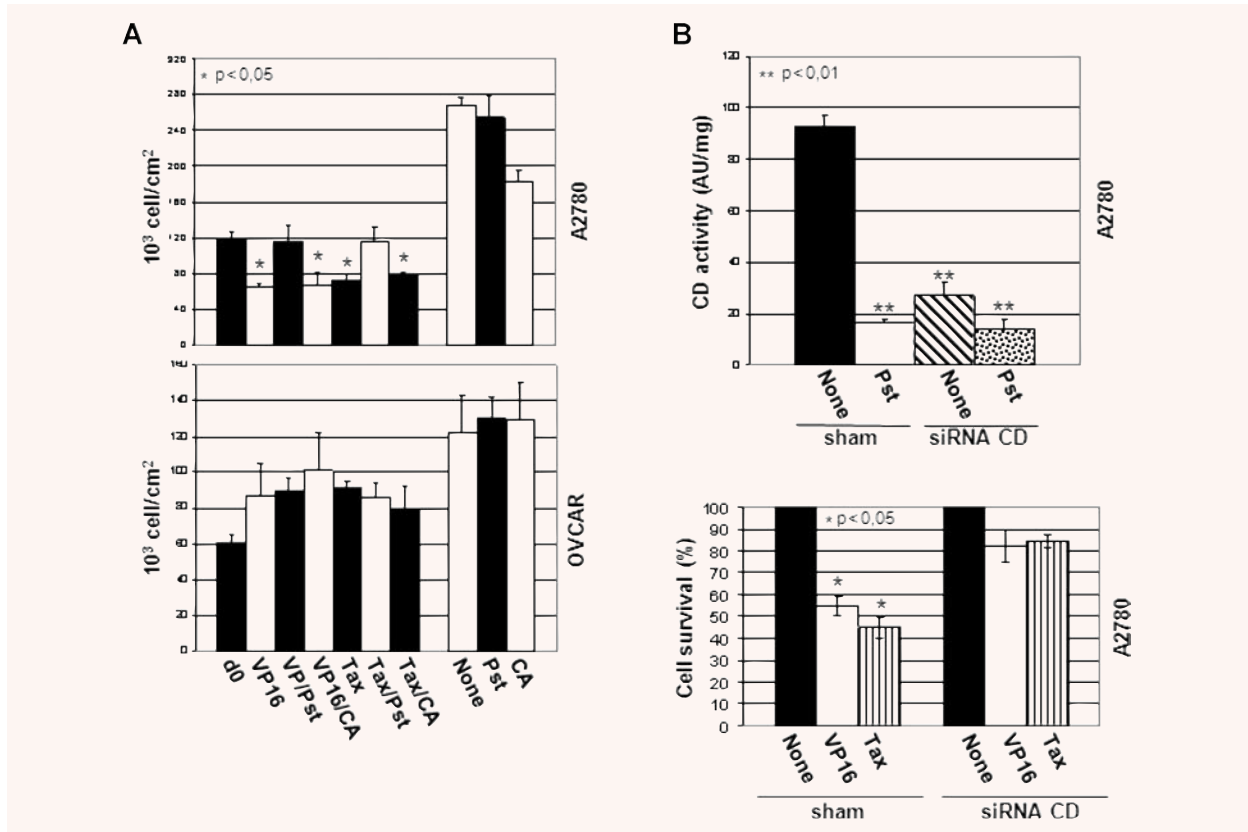
### **Inhibition of cathepsin D, not of cathepsins B or L, prevents cell death induced by chemotherapy drugs**

Bax-mediated permeabilization of mitochondria and chromatin alterations in VP16- and taxol-induced cell death (Fig. 1) are suggestive of the involvement of lysosomal cathepsins [19]. To assess the possible participation of the cysteine proteases CB and CL and/or the aspartic protease CD in this process we first employed the inhibitors CA074Me and Pst, which are rather specific for CB and CL, and for CD, respectively [42–44]. With respect to cell density at time zero, cell loss (amounting to approximately 50%) was observed in A2780 cultures exposed for 24 hrs to either VP16 or taxol. This cell loss was completely prevented by Pst, not by CA074Me (Fig. 3A). Final cell density in CA074Me-treated A2780 cultures was reduced compared to that in control untreated or only Pst-treated counterparts, indicating that inhibition of CB and CL was itself cytotoxic to some extent to these cells (Fig. 3A). OVCAR cells showed once again resistance to both chemotherapy drugs and also non-sensitive to the growth-inhibitory effect of CA074Me (Fig. 3A). Consistent with these data, in VP16-treated and in taxol-treated A2780 cells Pst prevented the occurrence of nuclei alterations, whereas CA074Me did not prevent such chromatin alterations and, unexpectedly, caused itself apoptosis to some extent as revealed by occurrence of chromatin fragmentation and mitochondrial leakage (not shown). In a separate experiment we used E-64d, another membrane permeable inhibitor of CB and CL [45], to further assess the involvement of these cathepsins in VP16- and taxol-induced apoptosis. 50  $\mu$ M E-64d showed

slightly cytotoxic in itself, and 10 and 20  $\mu$ M of this inhibitor did not rescue A2780 cells from chemotherapeutic drug-induced apoptosis (not shown). Pst is known to inhibit also cathepsin E, beside CD, another aspartyl protease that could also be implicated in chemotherapy-induced apoptosis. To definitely rule out the involvement of any aspartyl protease other than CD in the death pathway activated by VP16 and taxol in A2780 cells we specifically down-regulated the expression of this protease by transient transfection with a specific siRNA 21-mer duplex. As controls, parallel cultures were transfected with an inefficient duplex oligonucleotide not targeting CD mRNA (sham transfected). Proper conditions were assessed to achieve optimal down-regulation of CD expression, which was monitored by assaying the proteolytic activity at acid pH on a fluorogenic substrate. As shown in Fig. 3B (upper panel), siRNA transfection successfully down-regulated (>80%) CD expression based on the fact that residual activity in transfected cells was as low as that measured in the presence of Pst. Western blotting confirmed the efficient post-transcriptional silencing of CD in the transfected cells (not shown). A parallel set of cultures was used to estimate cell vitality upon treatment with chemotherapy drugs. In control duplex-transfected cultures cell viability was reduced of approximately 50% upon 24-hrs exposure to VP16 or taxol (Fig. 3B, lower panel). By contrast, in siRNA-transfected cultures the cytotoxic effect of both the chemotherapy drugs was negligible (Fig. 3B, lower panel). Cytofluorometry analysis of annexin V-labelled cells confirmed the protection by CD-siRNA against VP16 and taxol cytotoxicity (not shown). The fact that Pst and CD-siRNA elicited the same protective effect indicates that CD-mediated proteolysis is a determinant factor of the cytotoxic response to the above chemotherapy drugs.

### **Cathepsin D activity is essential for Bax-mediated permeabilization of mitochondria and apoptosis**

Bax has been shown to promote mitochondrial permeabilization and relocation of pro-apoptotic proteins from mitochondria into the cytosol [46]. In various paradigms of cytotoxic treatments conformational changes and oligomerization on mitochondrial membrane of Bax were shown to depend on CD [15, 28, 29]. We thus tested whether CD was implicated in Bax-dependent activation of the intrinsic death pathway in A2780 cells. Both Pst-mediated inhibition of CD activity and siRNA-mediated silencing of CD expression prevented the oligomerization of Bax and the mitochondria permeabilization induced by VP16 in A2780 (Fig. 4A and B). Caspase 3-mediated hydrolysis of poly(ADP-ribose) polymerase, an enzyme involved in DNA repair, results in the accumulation of nicked DNA in nuclei of apoptotic cells that can be demonstrated with the TUNEL technique. Thus, to definitely prove the involvement of CD in caspase 3 activation we checked for the presence of TUNEL-positive cells in A2780 cultures exposed to VP16 along with Pst. As expected in case of true caspase-dependent apoptosis, A2780 cells exposed to VP16 showed TUNEL positive (Fig. 4C). However, when the cytotoxic treatment was performed in the presence of Pst the



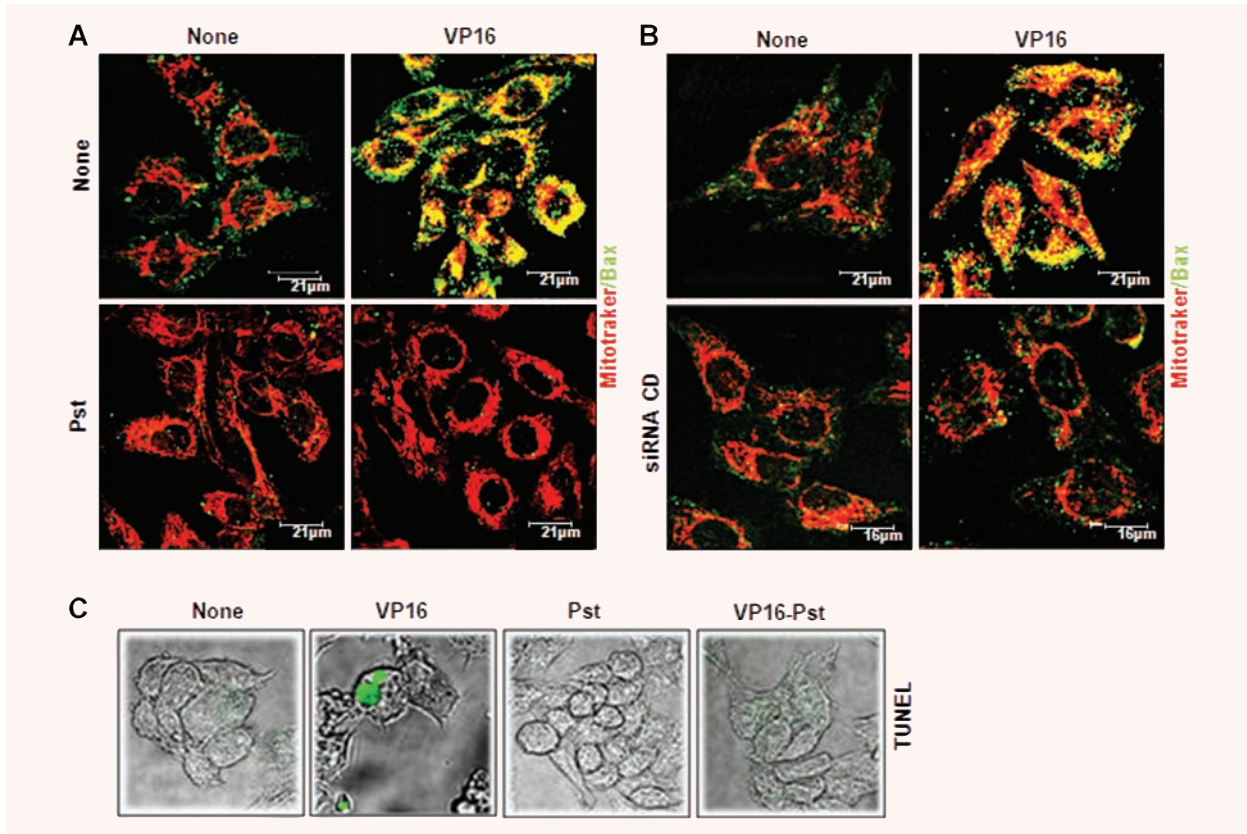
**Fig. 3** Cathepsin D, not cathepsin B, mediates chemotherapy drug toxicity in A2780 cells. **(A)** A2780 cells and OVCAR cells plated on Petri dishes were exposed or not for 24 hrs to VP16 or taxol (Tax) in the absence or the presence of the CD inhibitor Pst or the CB inhibitor CA074Me (CA). At the end, cells were harvested and counted. The experiment was repeated four times in triplicate. Data are given as mean  $\pm$  S.D. In A2780 cells (top panel) both VP16 and taxol induced cell loss, which was prevented by Pst not by Ca074Me (compare final density at day 1 in treated *versus* initial density at d0 in cultures; note that Pst abrogated cell death by the chemotherapy drugs though it not completely rescued the cells from a growth block). Inhibition of CB and CL, on the other hand, did not protect from drug toxicity and, on itself, caused some cell loss in A2780 culture. In OVCAR cells (bottom panel) VP16 and taxol were not toxic and no effect were exerted by the cathepsin inhibitors either alone or in combination with the chemotherapy drugs. **(B)** A2780 cells plated on Petri dishes were transfected with a control duplex RNA (sham) or a CD specific siRNA and 24 hrs later incubated in fresh medium for further 24 hrs in the absence or in the presence of VP16 or taxol (Tax). Prior to start the treatment, CD activity was assayed in parallel homogenates to ascertain down-regulation of CD in siRNA-transfected cultures (upper panel). At the end of the incubation, cell survival was estimated counting the adherent trypan-blue excluding cells (lower panel). Data (mean  $\pm$  S.D.) are given as percentage of controls and arise from three independent experiments.

cell monolayer appeared well preserved and nuclei did not stain for TUNEL (Fig. 4C), indicating that in these cells activation of caspase 3 was precluded. These data indicate that in chemotherapy drug-sensitive cells activation of the CD-mediated proteolytic pathway precedes that of the caspase cascade.

### Lysosome permeabilization, not cathepsin D expression, is the limiting factor for the activation of the intrinsic death pathway

Next, we investigated the molecular link between the proteolysis mediated by a lysosomal protease (*i.e.* CD) and the activation of a

cytosolic protein (*i.e.* Bax). First, we considered the possibility that chemoresistance in OVCAR cells could arise from abnormally low expression of CD. Yet, when analysed by Western blotting, the basal expression level of CD was found much higher (~15 folds) in OVCAR than in A2780 cells (Fig. 5A). On treatment with VP16 the expression of CD did not change in OVCAR cells, whereas it greatly increased (~10 to 15 folds at 24 and 48 hrs, respectively) in chemosensitive A2780 cells (Fig. 5A). Based on previous findings [15, 28, 29, 47, 48], we hypothesized that Bax activation followed the cytosolic translocation of CD upon cytotoxic treatments. We thus looked at the compartmentalization of CD in the two cell lines under cytotoxic treatment. To better assess the possible relocation of CD from lysosomes into the cytosol, we labelled the



**Fig. 4** Cathepsin D triggers Bax-mediated permeabilization of mitochondria and TUNEL-positive cell death in response to VP16. A2780 cells were let adhere on coverslips and pre-incubated for 16 hrs with Pst (A) or sham-transfected or transfected with a CD-specific siRNA (B). The cells were then treated or not with VP16 for 24 hrs and thereafter processed for mitotracker staining and Bax immunofluorescence. Both genetic down-regulation and pharmacological inhibition of CD prevented the activation and oligomerization of Bax onto mitochondria induced by VP16. (C) A2780 cells adherent on coverslips were exposed for 24 hrs to VP16 and then stained with the TUNEL technique that demonstrated the presence of nicked DNA in apoptotic cells. Images are representative of three separate experiments. Selected fields are shown. Note that only cells still adherent on coverslips were labelled (detached cells were discarded).

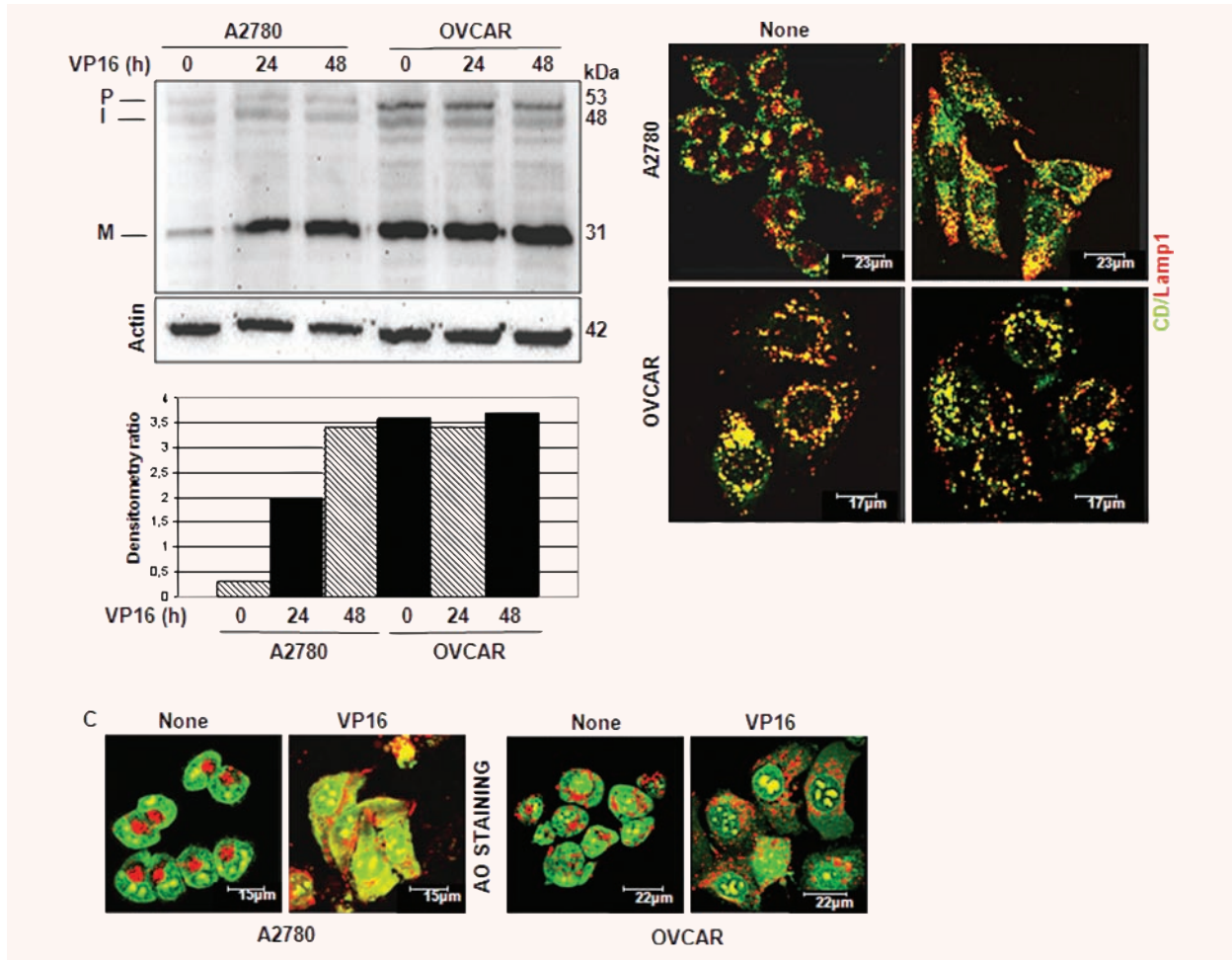
lysosomes with an antibody directed against Lamp1, an integral protein of lysosomal membrane. The images in Fig. 5B reveal that in VP16-treated A2780 cells the pattern of CD staining is diffuse as expected for cytosolic localization and does not show the typical punctate staining and co-localization with Lamp1, as would be expected for lysosomal localization and as it is seen in control untreated cells. By contrast, in OVCAR cells CD showed a punctate staining and full co-localization with Lamp1 regardless of the exposure to the cytotoxic drug. We finally tested whether changes in CD compartmentalization were the consequence of a generalized permeabilization of the lysosomal membrane. The integrity of lysosomes was checked by the AO retention test. AO is an acidotropic fluorochrome that once protonated is retained within lysosomes and emits red fluorescence. However, in the presence of micropores in the lysosomal membrane AO diffuses into the cytosol where it emits a green fluorescence [49]. OVCAR and

A2780 cells adherent on coverslip were exposed to VP16 and observed under the fluorescence microscope after AO staining. In control cells AO fluorescence appeared as intense red spots, indicating that it was confined within acid organelles (Fig. 5C). In OVCAR cells the AO fluorescence pattern was not affected by the cytotoxic treatments. By contrast, in treated A2780 cells a diffuse AO green-like fluorescence, indicative of cytosolic localization of the fluorochrome, was observed (Fig. 5C).

### **Bax mediates the permeabilization of lysosomes induced by VP16 in chemosensitive A2780 cells**

How lysosomal membrane is permeabilized, thus allowing cathepsins to escape into the cytosol, following the treatment with a cytotoxic drug remains elusive. It has been proposed that members

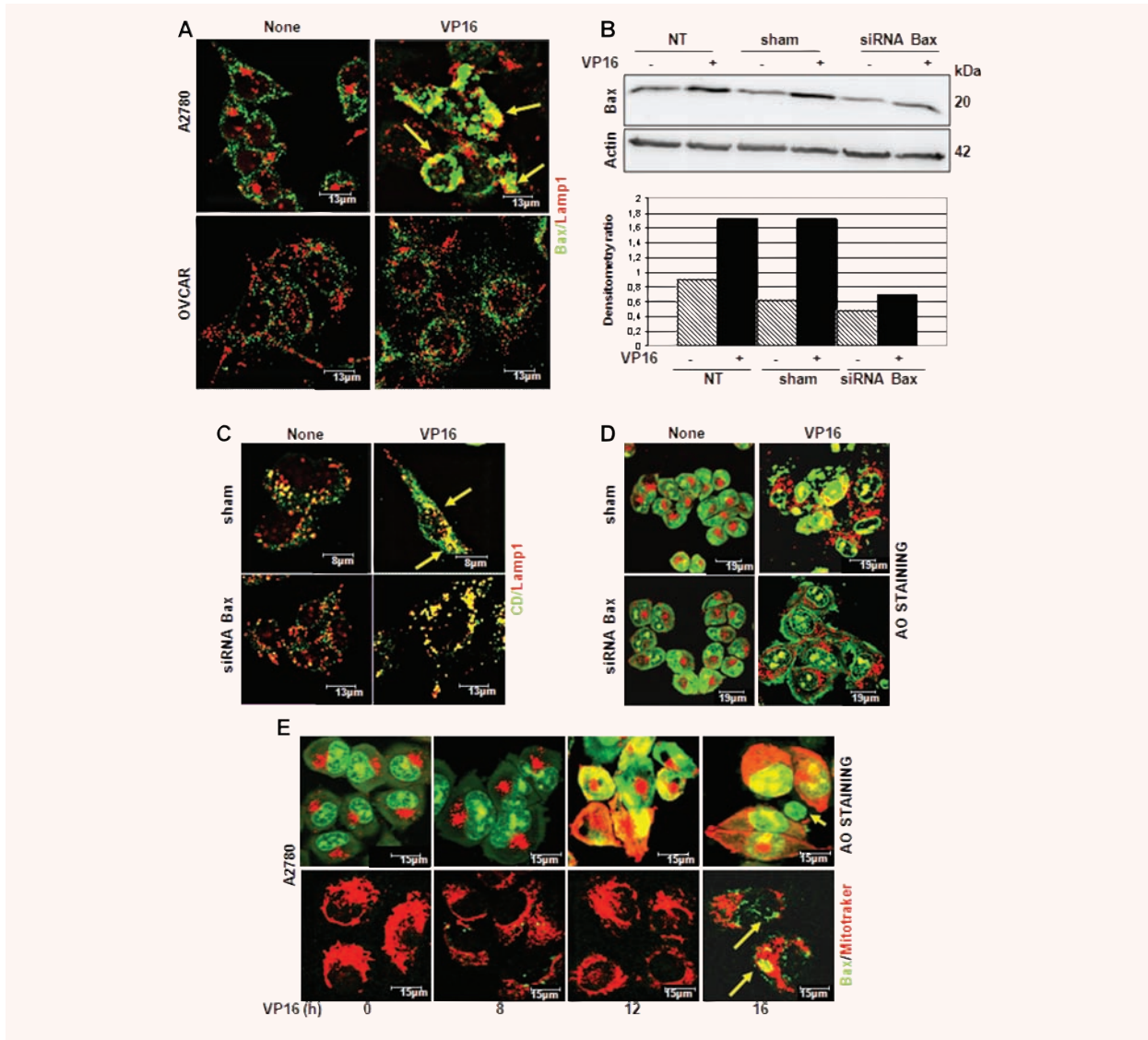




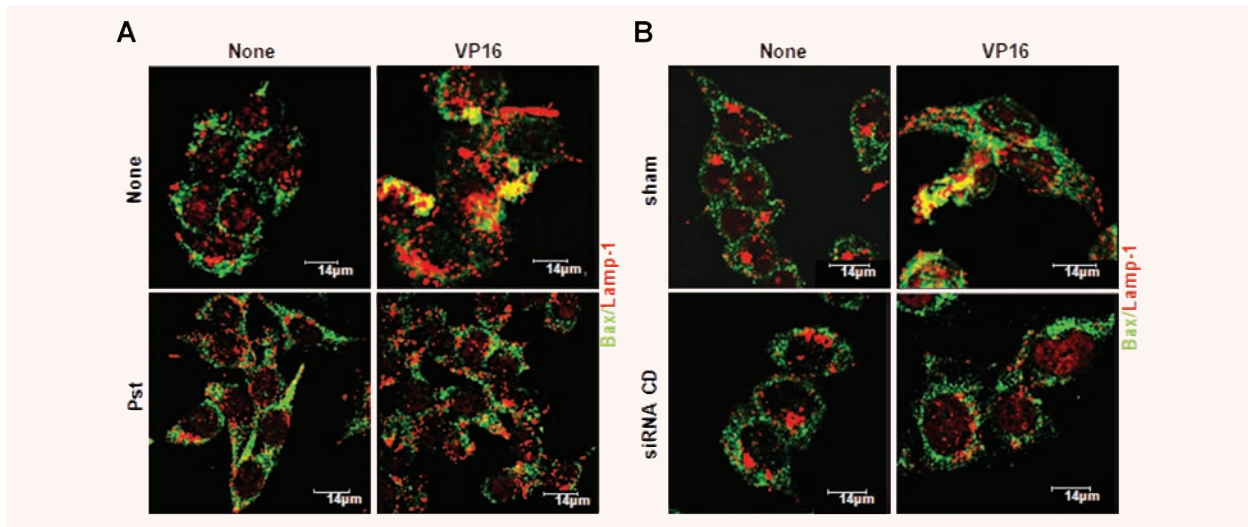
**Fig. 5** Effects of VP16 on the expression and localization of cathepsin D and on lysosome integrity. **(A)** Western blotting analysis of CD expression in A2780 and OVCAR cells. The precursor (P) and the mature double-chain form resident in lysosomes (M, only the large chain is visible on gel) are expressed at different ratio in the two ovarian cancer cell lines. The chemoresistant OVCAR cell line basally expresses higher level of both proCD and mature CD than the chemosensitive A2780 cell line. VP16 greatly increases the cellular content of mature CD in A2780 cells, whereas it has no effect on CD expression in OVCAR cells (densitometry of the M polypeptide normalized against actin is shown). **(B)** Cells adherent on coverslips were treated for 24 hrs with VP16 and then processed for immunofluorescence staining of CD (green fluorescence) and Lamp1 (red fluorescence). The images show a diffuse CD staining indicative of cytosolic localization in VP16-treated A2780 cells (lysosomes are identified as Lamp1-positive vesicles), whereas in control untreated cells CD staining appears as definite yellow spots that denote its localization within Lamp1-positive vesicles. In OVCAR cells, either control or VP16-treated, CD shows a granular-like staining and fully co-localizes with Lamp1. **(C)** Cells adherent on coverslips and exposed or not to VP16 were stained with acridine orange (AO) and observed under the fluorescence microscope. Red staining is indicative of segregation of the fluorochrome within acid compartments (*i.e.* endosomes and lysosomes), whereas green fluorescence is indicative of cytosolic localization of the fluorochrome. The images demonstrate that in A2780 cells, not in OVCAR cells, VP16 induces the cytosolic relocation of AO. The results shown in **(A)**, **(B)** and **(C)** are representative of at least three independent experiments.

of the bcl-2 family could also affect the stabilization of lysosomal membranes [50]. Consistently, Bax has been shown to insert onto lysosomal membranes in staurosporine-treated fibroblasts [51]. We found that on treatment with VP16, Bax in fact was activated and formed macro-aggregates positive also for Lamp1 in A2780 cells, but not in OVCAR cells (Fig. 6A). To see whether lysosomal

relocation of Bax was functionally related to the permeabilization of lysosomes in VP16-treated A2780 cells, we specifically down-regulated the expression of Bax in these cells and looked at the compartmentalization of CD and at lysosome integrity. Bax gene expression in A2780 cells was efficiently silenced (~85%) after a 24-hrs transfection with a specific siRNA (Fig. 6B). Under such



**Fig. 6** Bax is essential for VP16-induced permeabilization of lysosomal in A2780 cells. **(A)** Cells adherent on coverslips were treated for 24 hrs with VP16 and then processed for immunofluorescence staining of Bax (green fluorescence) and Lamp1 (red fluorescence). The images show a diffuse and faintly detectable Bax staining in control A2780 cells, as well as in control and VP16-treated OVCAR cells. The granular-like staining of Lamp1 identifies the lysosomes. Of note, in VP16-treated A2780 cells Bax appears as intense yellow spots indicative of conformational activation and aggregation on Lamp1-positive vesicles. **(B)** A2780 cells plated on Petri dishes were sham- or Bax-siRNA-transfected or not transfected (NT) and after 24 hrs exposed or not for further 24 hrs to VP16. The Western blotting of Bax confirms that: (i) VP16 induced the expression and accumulation of Bax; (ii) siRNA transfection efficiently silenced the expression of Bax (see densitometry). **(C)** A2780 cells adherent on coverslips were sham- or Bax-siRNA-transfected and after 24 hrs exposed or not to VP16 for further 24 hrs. At the end, cells were fixed and processed for immunofluorescence staining of CD (green fluorescence) and Lamp1 (red fluorescence). The images show a complete co-localization of CD and Lamp1 in sham-transfected untreated cells, whereas CD appears relocated in the cytosol upon treatment with VP16. However, in Bax-silenced cells CD staining coincides with that of Lamp1 regardless of whether they had been exposed to VP16. **(D)** A2780 cells adherent on coverslips and transfected and incubated as for the experiment described in **(C)** and then stained with acridine orange (AO). The fluorescence images demonstrate that genetic silencing of Bax precludes the lysosomal permeabilization induced by VP16. **(E)** Time-course analysis of lysosome leakage and Bax-mediated mitochondrial permeabilization. Images demonstrate that permeabilization of lysosomes (upper panel, 12 hrs) precedes that of mitochondria (arrows in lower panel, 16 hrs). Apoptotic cells become apparent at 16 hrs (arrow in upper panel). The results shown in this figure are representative of at least three separate experiments.



**Fig. 7** Cathepsin D drives the Bax activation and relocation onto lysosomes on VP16 treatment. Cells adherent on coverslips were either sham- or CD-siRNA-transfected as for the experiments described in Fig. 4(B). Parallel coverslips were pre-incubated or not with Pst (A). The cells were then exposed or not for 24 hrs to VP16 and thereafter processed for immunofluorescence staining of Bax (green fluorescence) and Lamp1 (red fluorescence). Bax staining is faintly detectable and diffused in the cytoplasm of untreated cells, whereas it appears as intense yellow spots, indicative of co-localization with Lamp1 (arrows), in sham-transfected and in Pst-non-preincubated A2780 cells exposed to VP16. Both siRNA-mediated down-regulation or Pst-mediated inhibition of CD prevented the Bax translocation onto lysosomes in VP16-treated cells, as demonstrated by the lack of co-localization with Lamp1. In these cells Bax staining is faintly detectable and diffused in the cytoplasm as in control untreated cells.

conditions, VP16-induced cytosolic relocation of CD (Fig. 6C) and of AO (Fig. 6D) did not occur in A2780 cells, proving that Bax relocation onto lysosomes is a determinant event for lysosomal membrane permeabilization. To better assess the functional link between lysosome leakage and Bax-mediated mitochondria permeabilization, we monitored these two events in a time-course treatment of A2780 cells with VP16. Lysosome leakage was not apparent prior to 12 hrs of exposure to the chemotherapeutic drug and involved approximately 50% of the cell population by 16 hrs, at which time-point some apoptotic cells became visible in the monolayer (Fig. 6E, upper panels). Bax oligomerization and mitochondria permeabilization, on the other hand, became apparent at 16 hrs (Fig. 6E, lower panels), indicating that apoptosis started at this time-point.

### Cathepsin D triggers the translocation of Bax onto lysosome membrane upon VP16 treatment

Since Bax activation and oligomerization on mitochondria was proven to depend on CD activity (see Fig. 4A and B and [28, 29]), we wondered whether CD was also implicated in Bax activation and relocation on lysosomal membranes. We thus looked at Bax localization in VP16-treated cells in which CD was either inhibited by pre-loading with Pst or post-transcriptionally down-regulated by siRNA transfection. The Bax-Lamp1 co-localization results

shown in Fig. 7 clearly demonstrate that CD activity is essential to induce the activation of Bax and its oligomerization onto lysosomes under chemotherapy drug treatment. These data suggest the existence of a positive loop of activation between CD and Bax that amplifies the death signal and leads to a generalized permeabilization of lysosomes and mitochondria, which can be assumed as the point of no return in the apoptotic process.

## Discussion

Resistance to chemotherapy drugs is the principal factor limiting long-term treatment success in ovarian cancer management [4]. The molecular dissection of the pathways activated by cytotoxic drugs may help to identify the molecular mechanisms implicated in multidrug resistance, and can positively reflect on designing new therapeutic approaches to circumvent this phenomenon [5]. Among the factors that concur to the resistant phenotype in cancer cells are the overexpression of ATP-dependent transporters that actively extrude the cytotoxic drugs [52] and defective activation of the apoptotic pathway due to altered expression of key regulators or executioners [12, 21, 31, 34]. Mutation in the oncosuppressor p53 and deficient expression of Bax have been associated with chemoresistance and poor prognosis in ovarian

cancers [31, 37, 53]. The present study focused on the cytotoxic mechanisms of VP16 and taxol, two drugs currently employed for the cure of ovarian cancer refractory to platinum-based chemotherapy [4, 25]. We dissected the apoptotic pathway activated by these drugs in two ovarian cancer cell lines, namely the A2780 cell line known to express only the wild-type form of p53 [26] and the OVCAR cell line that express a functionally inactive mutant form of p53 [27]. A2780 cells were shown to be far more sensitive than OVCAR cells in response to both VP16 and taxol, and also to *cis*-platinum and resveratrol. The ratio of Bax to bcl-2 expression in the cell is a critical determinant of the threshold at which apoptosis is induced by chemotherapy drugs [33]. Accordingly, Bax was highly expressed and bcl-2 faintly detectable in A2780, whereas bcl-2 was highly expressed and Bax faintly detectable in OVCAR cells. Drug cytotoxicity in A2780 cells presented as TUNEL-positive cell death and was associated with the Bax-mediated permeabilization of mitochondria. It is to note that this event was temporally preceded by lysosome permeabilization, which allowed the cytosolic relocation of CD and, very likely, of other lysosomal cathepsins. Under cytotoxic treatments, lysosomal cathepsins B, L and D have been shown to induce mitochondrial permeabilization by targeting and activating either bid or Bax [15–17, 20–22, 28, 29, 45, 54]. The participation of CB and CL in this process was, however, excluded based on the use of CA074Me and E-64d inhibitors. In the present paradigm, pharmacological inhibition and siRNA-mediated down-regulation studies clearly demonstrated that activation and relocation of Bax were dependent on CD activity. The interaction of CD with Bax is likely to occur in the cytosol, where the lysosomal protease is in fact found upon treatment with the chemotherapy drugs. This raises the important issues (i) as whether CD can be active at the cytosolic pH and (ii) about the mechanism linking CD proteolysis to Bax activation. CD has been shown able to perform a limited (activating) proteolysis on the precursor of the parathyroid hormone within the endosome at pH ~6.5 [55], and it is known that the cytosolic pH of cells undergoing apoptosis drops to 6.8–5.7 [56]. More recently, we have demonstrated that CD affects a limited proteolysis on prolactin at physiological pH, which results in the generation of bioactive peptides [57, 58]. Therefore, it is conceivable that mature CD can act on small substrates at the cytosolic pH in cells undergoing apoptosis. It has been reported that cytosolic acidification is mandatory for mitochondrial translocation of Bax during drug-induced apoptosis of tumour cells [59]. We hypothesize that CD triggers the conformational activation of Bax by either affecting a limited proteolysis on Bax itself (though no changes in the apparent molecular weight of Bax was observed in our experiments) or, more likely, by proteolytic inactivation of a Bax-interacting chaperone that prevents Bax activation. One such candidate could be clusterin, which is highly expressed in malignant tumours and specifically binds to Bax in response to chemotherapeutic drugs [60]. Alternatively, CD proteolysis facilitates the binding of Bax with a chaperone (e.g. nucleophosmin) that promotes its translocation onto mitochondria [61].

Another important and original finding of the present study is that the permeabilization of lysosomes follows the activation and oligomerization of Bax onto lysosomal membranes and that CD activity is essential for such an event. This demonstrates the existence of an activation loop between CD and Bax that serves to recruit an increasing number of Bax molecules. With time, the number of active Bax molecules will exceed the number of bcl-2 and bcl-X<sub>L</sub> molecules available for heterodimerization, so that free active Bax molecules can start to oligomerize on mitochondrial membrane. This event will mark the 'point of no return', as it will determine the release of cytochrome c and other pro-apoptotic factors that will finally form the apoptosome. We propose the following model of toxicity of pro-apoptotic drugs: (i) a CD-mediated proteolysis of a so far unknown substrate leads to the activation of Bax and in turn to Bax-mediated permeabilization of lysosomes and consequent cytosolic relocation of cathepsins; (ii) with time, active Bax molecules reach a number sufficient to allow their dimerization on mitochondria and (iii) the permeabilization of mitochondria allows the cytosolic relocation of factors needed for the formation of the apoptosome and consequent activation of the caspase cascade. It is to note that the chemotherapy drug treatment induced lysosomal permeabilization in A2780, not in OVCAR cells. Chemoresistance of p53-defective OVCAR cells could be due to: (i) insufficient accumulation of the drug within the cell (possibly because of the overexpression of outward transporters) or (ii) inability to rise up the cellular levels of Bax and/or CD that are, in fact, transcriptionally targeted by p53 [21, 62] or (iii) other unknown factors. However, it can be assumed that VP16 reached in OVCAR cells a concentration sufficient for signalling as it caused a marked up-regulation of p53 and a concomitant strong down-regulation of bcl-2 expression. Also, unlikely CD activity in OVCAR cells was not sufficient to start the apoptotic process, as the basal expression of CD in these cells was by far much higher than in A2780 cells. We rather suspect that the inability to initiate the permeabilization of lysosomes in chemoresistant OVCAR cells arises from insufficient Bax expression. In conclusion, we have shown that the cooperation between CD and Bax is essential to determine the permeabilization of lysosomes and that this represents the primary and limiting factor that determines the responsiveness to chemotherapy drugs. Forcing the leakage of cathepsins from lysosomes could be a strategy to overcome chemoresistance, provided that the down-stream apoptotic machinery is intact.

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